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UNIVERSITY OF ALBERTA

# Studies on Transfected Hepatoma Cell Lines Expressing Recombinant Human Apolipoprotein B

BY

**Sherri Lynn Selby**

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**.

DEPARTMENT OF BIOCHEMISTRY

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ABSTRACT

Human apolipoprotein (apo) B is necessary for the assembly and secretion of hepatic triglyceride-rich lipoproteins. Investigation of naturally occurring carboxyl terminally truncated human apoB variants suggests that the carboxyl terminal deletion of human apoB reduces both size and secretion of hepatic triglyceride-rich apoB-containing lipoproteins. To examine the effect of carboxyl terminal deletion of apoB in the assembly and secretion of hepatic lipoproteins, stable transformants of the rat hepatoma cell line McArdle rat hepatoma 7777 (McA-RH7777) that synthesize and secrete carboxyl truncated forms apoB64 and apoB72 (representing the amino terminal 64 and 72 % of apoB100) as well as the full length apoB100 protein were generated. The apoB proteins secreted into the medium were fractionated by density gradient ultracentrifugation and detected by immunoblotting. Combined with data from previous work (Yao Z, Blackhart BD, Linton MF, Taylor SM, Young SG, McCarthy BJ, 1991 *J Biol Chem* 266:3300-3308; McLeod RS, Zhao Y, Selby S, Westerlund J, Yao Z, 1994 *J Biol Chem* 269:2852-2862), the present results revealed that the particle density was inversely related to the length of apoB from apoB23 to apoB53 (residues 1003 - 2377) and from apoB64 to apoB90 (residues 2880 - 4082), suggesting that these two regions of apoB play important roles in apoB-containing lipoprotein assembly. The efficiency and rate of secretion of these truncated forms of apoB were determined by pulse chase analysis. The results indicated that carboxyl-terminal truncation does not impair apoB secretion: apoB64 and apoB72 were secreted as efficiently as rat apoB100.

Overall, these data imply that the size of apoB governs the amount of lipid to be secreted in an apoB-containing lipoprotein and that low apoB levels observed *in vivo* may not be caused by impaired secretion of apoB proteins.

The effect of apoB mRNA level on hepatic apoB production has not been studied extensively since the steady state level of apoB mRNA can not be altered acutely. To determine the effect of vastly different apoB mRNA levels on the synthesis and secretion of apoB-containing lipoproteins, McA-RH7777 cell lines stably transfected with cDNA constructs encoding human apoB53 (representing 53 % of the full length apoB100) were obtained that expressed different levels of apoB53 mRNA. Immunoblotting demonstrated that the steady state levels of intracellular and secreted apoB53 protein positively correlated with apoB53 mRNA levels. The level of expression of apoB53 did not affect the buoyant density of lipoproteins containing apoB53 (d=1.06 - 1.21 g/ml) or endogenous rat apoB100 (d<1.06 g/ml) nor did it affect the level of secretion of endogenous rat apolipoproteins (*e. g.*, apoAI). [$^{3}$H]glycerol incorporation and lipid mass analysis demonstrated that in cells producing high levels of apoB53, triglyceride synthesis (2-fold) and secretion (2- to 3-fold) were both increased while a 25 % reduction in the triglyceride cellular pool was observed, relative to the low expressor. [$^{35}$S]methionine incorporation and pulse chase experiments indicated that between cell lines containing high or intermediate levels of apoB53 mRNA, there was an 8-fold difference in synthesis and a 2-fold increase in the secretion efficiency of apoB53 protein. In total, these data suggest that the level of apoB mRNA has an important effect on the synthesis and secretion of apoB-containing lipoproteins.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| a. a. | amino acid |
| abeta | abetalipoproteinemia |
| AF-1 | apolipoprotein factor-1 |
| apo | apolipoprotein |
| ARP-1 | apolipoprotein response protein-1 |
| BBS | BES buffered saline |
| BES | N,N-bis-(hydroxy-ethyl)-2-amino-ethanesulfonic acid |
| BPB | bromophenol blue |
| BSA | bovine serum albumin |
| CE | cholesteryl ester |
| C/EBP | CCAT/enhancer binding protein |
| CHO | Chinese hamster ovary cells |
| COS-7 | CV-1 origin, SV40 transformed monkey kidney cell line |
| d | density |
| DMEM | Dulbecco's modified Eagle's medium |
| DTT | dithiothreitol |
| EAR-2 | v-erbA related human receptor-2 |
| EAR-3 | v-erbA related human receptor-3 |
| ECL | enhanced chemluminescence |
| EDTA | ethylenediaminetetraacetate |
| ER | endoplasmic reticulum |

| | |
|---|---|
| FC | free (unesterified) cholesterol |
| HDL-2 | high density lipoprotein-2 |
| HDL-3 | high density lipoprotein-3 |
| HNF-4 | hepatic nuclear factor-4 |
| HPTLC | high performance thin layer chromatography |
| hypobeta | hypobetalipoproteinemia |
| hyperapobeta | hyperapobetalipoproteinemia |
| IDL | intermediate density lipoprotein |
| IgG | immunoglobulin G |
| LCAT | lecithin:cholesterol acyl transferase |
| LDL | low density lipoprotein |
| LPL | lipoprotein lipase |
| McA-RH7777 | McArdle rat hepatoma 7777 |
| MTP | microsomal triglyceride transfer protein |
| n= | number of samples |
| ND | not determined |
| NF-BA1 | nuclear factor-BA1 |
| nt | nucleotide |
| p27 | protein of 27 kD involved in apoB mRNA editing |
| PBS | phosphate buffered saline |
| PC | phosphatidylcholine |
| PDI | protein disulfide isomerase |

| | |
|---|---|
| PEST | sequences within a protein rich in proline, glutamic and aspartic acid, serine and threonine flanked by clusters of positively charged amino acids |
| PL | phospholipid |
| PMME | phosphatidylmonomethylethanolamine |
| PMSF | phenylmethanesulfonyl fluoride |
| RIPA | radioimmune precipitation |
| S. D. | standard deviation |
| SDS | sodium dodecyl sulfate |
| SDS PAGE | SDS polyacrylamide gel electrophoresis |
| SRP | signal recognition particle |
| $t_{1/2}$ | intracellular retention half time |
| TBS | Tris buffered saline |
| TC | total cholesterol |
| TG | triglyceride |
| TLC | thin layer chromatography |
| Tween 20 | poly(oxyethylene)$_{20}$-sorbitan-monolaurate |
| v | volume |
| VLDL | very low density lipoprotein |
| w | weight |
| A | amperes |

| | |
|---|---|
| b | base |
| $^{o}C$ | degrees Celsius |
| Ci | curies |
| cpm | counts per minute |
| D | daltons |
| dpm | disintegrations per minute |
| g | grams |
| l | liters |
| M | moles/liter |
| N | normal |
| rpm | revolutions per minute |
| S | svedbergs |
| V | volts |

(standard prefixes are: p, pico; μ, micro; m, milli; k, kilo)

| | |
|---|---|
| A | adenine |
| C | cytosine |
| G | guanine |
| T | thymine |
| U | uracil |

| | |
|---|---|
| asn | asparagine (N) |
| asp | aspartic acid (D) |
| cys | cysteine (C) |

| | |
|---|---|
| glu | glutamic acid (E) |
| gln | glutamine (Q) |
| leu | leucine (L) |
| pro | proline (P) |
| ser | serine (S) |
| thr | threonine (T) |

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# Chapter I. INTRODUCTION

## I.1. PHYSICOCHEMICAL PROPERTIES OF PLASMA LIPOPROTEINS

Plasma lipoproteins are large, spherical complexes that consist of a hydrophobic core of triglycerides and cholesteryl esters surrounded by an amphipathic monolayer of phospholipid and unesterified cholesterol stabilized by specific proteins called apolipoproteins. Since lipoproteins have a lower buoyant density than most plasma proteins owing to the presence of lipids, lipoproteins can be readily isolated from plasma by ultracentrifugation. The buoyant density of lipoprotein particles is inversely related to their size, reflecting the relative amounts of nonpolar core lipid and high density, surface protein present. Lipoproteins are usually separated into six main classes based on buoyant density: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoprotein-2 ($HDL_2$) and high density lipoprotein-3 ($HDL_3$). Some of the physical and chemical properties of these lipoproteins are summarized in Table I.1. Each class is heterogeneous with respect to size and composition.

Chylomicrons are by far the largest of the lipoprotein classes; they are synthesized by enterocytes and contain dietary triglycerides and cholesterol as their major lipid constituents. VLDL are the next largest class of lipoproteins and are produced by hepatocytes; they are responsible for delivering endogenously synthesized lipid from the liver to extrahepatic cells. While chylomicrons and VLDL are specialized primarily for the

transport of triglycerides, LDL and HDL carry cholesterol and cholesteryl esters as their predominant lipid component. The mature forms of LDL and HDL are not secreted directly from cells but rather are produced by metabolic processes within the blood plasma. IDL represent the intermediates of VLDL lipolysis by lipoprotein lipase with LDL as the metabolic endproduct. Protein components of HDL are secreted with chylomicrons and VLDL or free of lipid (for review see Davis 1991, Fielding and Fielding 1991).

## I.2. MAJOR APOLIPOPROTEINS

The protein components of plasma lipoproteins are called apolipoproteins. Apolipoproteins (apo) perform several functions such as stabilizing lipoprotein particles (*e. g.*, apoB100, apoB48), serving as cofactors for lipolytic enzymes (*e. g.*, apoCII), or as receptor ligands (*e. g.*, apoE) that permit uptake of the lipid-protein complexes by the cell. There are essentially two types of apolipoprotein: exchangeable apolipoproteins such as apoAI and apoE which can transfer from one lipoprotein particle to another, and nonexchangeable apolipoproteins, such as apoB100 and apoB48, which can not. Some properties of the major apolipoproteins are summarized in Table I.2. For example, human apoAI is a 28 kD protein synthesized primarily by the liver and intestine. ApoAI is one of the most abundant apolipoproteins in plasma (100 - 130 mg apoAI/dl plasma), and is a major component of HDL. ApoAI was the first apolipoprotein for which the cDNA was cloned; its gene was mapped to chromosome 11 and found to be closely linked to the genes for apoCIII and apoAIV (Zannis *et al.* 1993).

Finally, apoAI is an effective activator of lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible for the formation of cholesteryl esters in plasma.

Nonexchangeable apolipoproteins consist of two apoB proteins, apoB100 and apoB48. ApoB100 refers to the full length protein while apoB48 represents the amino-terminal 48 % of the full length protein. ApoB100-containing lipoproteins are synthesized in the human liver and are required for the transport of endogenously derived triglyceride and cholesterol in the circulation (see section I.3.). ApoB48 is synthesized in the intestine in man and is necessary for the absorption and distribution of dietary lipid (see section I.4.).

## I.3. APOLIPOPROTEIN B100

### I.3.1 Structure of ApoB100

Human apoB100 is one of the largest monomeric proteins secreted. Mature apoB100 consists of 4536 amino acids and has a core molecular weight of 512 kD (550 kD with glycosylation). The protein is unique among the apolipoproteins because of its size and some of its structural features (see below), as well as its insolubility in aqueous media upon delipidation.

#### I.3.1.1. Conformation of ApoB100

The conformation of apoB100 on lipoproteins is not known in detail. It has been proposed, based on electron microscopy (Phillips and Schumaker 1989, Chatterton *et al.* 1991) and protease treatment of LDL (Yang *et al.*

1986 and 1989), that apoB100 extends over the surface of the particle with multiple regions linked by flexible chains (Figure I.1). Reactivity of human apoB100 to monoclonal antibodies varies between the classes of lipoproteins that apoB100 is associated with, suggesting conformational changes occur depending on the state of lipidation (Chan 1992; references cited within).

I.3.1.2. Structural Features of ApoB100

I.3.1.2.1. Hydrophobic (Lipid Binding) Regions

The complete primary sequence of apoB100 has been established from overlapping cDNA clones and from protein sequencing (Yang *et al.* 1989; references cited therein). Sequence analysis reveals that apoB100 has many hydrophobic regions distributed throughout its length. Notably, none of these hydrophobic regions is long enough to constitute a 20 amino acid membrane-spanning region characteristic of an integral membrane protein. Nevertheless, these hydrophobic regions are believed to be important for lipid binding (Olofsson *et al.* 1987).

Use of an algorithm on secondary structure predicts that apoB100 contains extensive $\alpha$ helices and $\beta$ strands. ApoB100 may also contain regions of 22 amino acids that form amphipathic $\alpha$ helices characteristic of the putative lipid binding regions of the exchangeable apolipoproteins. These structures occur primarily within the center (residues 2035 - 2506) and carboxyl-terminal half of the apoB100 molecule (residues 4002 - 4527) (Knott *et al.* 1986, De Loof *et al.* 1987). The frequency of $\alpha$ helices in

apoB100, however, is much lower than the frequency observed in the exchangeable apolipoproteins. Instead, amphipathic β strands that may form amphipathic β sheets are probably more characteristic of apoB100. The β strands usually consist of 5 amino acid repeats (consensus sequence: acidic - aromatic - polar - aliphatic - proline) with a variable number of spacer amino acids between the repeats. These structures are clustered in four proline-rich regions (residues 1167 - 3865). The proline residues may confer a turn (β turn) in the amino acid sequence that allows the β strands to run in reverse orientation to one another, forming antiparallel β sheets (Knott *et al.* 1986). The amphipathic β sheets are unique to apoB100 and are thought to have high lipid binding potential. Together with the amphipathic α helices, the β strands may be important for the assembly of apoB100-containing lipoproteins (discussed below).

More direct proof of the involvement of these regions of apoB100 in lipid binding comes from incubating proteolytic fragments of apoB100 with microemulsions of exogenous lipids and isolation of the lipid-peptide complexes by ultracentrifugation (Chan 1992; references cited therein). Moreover, synthetic β strands organize lipids into LDL-like particles and bind tightly to plasma LDL (Chen *et al.* 1989). Similarly, studies with naturally occurring carboxyl-terminal truncated forms of apoB100 or with the recombinant apoB forms expressed in cell culture indicate that the lipid binding regions are distributed throughout apoB100 such that the length of apoB100 determines the density and size of the lipoproteins formed (Young 1989, Linton *et al.* 1993a, Yao *et al.* 1991, McLeod *et al.* 1994). One of the

objectives of this thesis is to determine the role of the carboxyl terminus of apoB100 in lipoprotein assembly and secretion.

I.3.1.2.2. LDL Receptor-Binding Regions

One of the major functions of apoB100 is to serve as a ligand for the LDL receptor. ApoE also serves as a ligand for the LDL receptor. Acidic residues in the amino-terminus of the LDL receptor are thought to interact with basic residues on the receptor-binding regions of apoB100 and apoE (Brown and Goldstein 1986). In apoE the receptor-binding region maps to residues 140 - 150 (Mahley 1988). ApoB100 has two regions rich in basic amino acids similar to the receptor-binding region of apoE (Knott *et al.* 1986). These regions span residues 3147 - 3157 (domain A) and 3359 - 3367 (domain B) of the apoB100 primary amino acid sequence. A synthetic oligopeptide corresponding to residues 3345 - 3381 of apoB100 conferred receptor-binding ability to trypsin-inactivated hypertriglyceridemic VLDL, permitted uptake of the VLDL by human fibroblasts and suppressed cellular 3-hydroxy-3-methylglutaryl coenzyme A reductase, an enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis (Yang *et al.* 1986). Sequence comparison among apoB100 from multiple vertebrate species suggests that while domain B maintains a strong net positive charge and shows high homology across species, domain A is not as well conserved with wide differences in net positive charge (Law and Scott 1990). The position of domain A and B matches the general position of the apoB100 receptor-binding region defined by monoclonal antibody mapping (residues 3000 - 4000) (Marcel *et al.* 1987, Milne *et al.* 1989, Pease *et al.* 1990).

Other regions of apoB100 may play an important part in LDL receptor-binding. Individuals with the syndrome of familial defective apoB100 have normal LDL receptors but elevated plasma cholesterol because their LDL have impaired receptor-binding ability (Innerarity *et al.* 1990). Genetic analysis demonstrated autosomal codominant transmission of the defect (Innerarity *et al.* 1990). DNA sequence analysis of the defective apoB allele indicated a G to A transition occurs at nucleotide position 10708 of the apoB cDNA (Soria *et al.* 1989). This substitution results in a change in the codon specifiying amino acid 3500 whereby a glutamine (CAG) replaces an arginine (CGG) at this position. The exact mechanism by which this mutation influences LDL receptor-binding is not known although it is thought that local conformational changes in and around the receptor-binding region may occur (Innerarity *et al.* 1990; references cited within). Studies with naturally occurring carboxyl-terminal truncations of apoB100 also suggest other regions remote from the receptor-binding site may serve to modulate receptor-binding activity (Parhofer *et al.* 1990, Krul *et al.* 1992).

I.3.1.2.3. Cysteines

Cysteine residues are important in stabilization of the tertiary structure of proteins. In apoB100 there are 25 cysteine residues; sixteen of them are involved in disulfide linkages, of which 12 occur in the first 500 amino acids (Figure I.2 A.). This uneven distribution suggests that the amino terminus is more globular in structure than the rest of the molecule (Yang *et al.* 1989). Except for the two amino-terminal cysteine pairs (cys-12 - cys-61, cys-51 - cys-70), disulfide linkages form between immediately neighboring cysteines.

Cysteine- 1085, 1395, 1478, 1635, 3734, and 3890 exist in reduced form while the state of cysteine 2906 and 4326 are unknown (Chan 1992).

I.3.1.2.4. Covalent Modifications

ApoB100 undergoes several covalent modifications including glycosylation, acylation and phosphorylation. There are 19 potential N-linked glycosylation sites (consensus sequence: Asn - X - Thr/Ser; X denotes any amino acid except proline) of which 16 were found to be glycosylated. These sites are distributed unevenly throughout the apoB100 sequence with a cluster of 8 within a 700 residue stretch between Asn-2752 and Asn-3438 (Yang *et al.* 1989, Chan 1992) (Figure I.2 B.). It has been observed that acylation of cysteine residues (Hoeg *et al.* 1987, Huang *et al.* 1987) and phosphorylation at serine and tyrosine residues occur within the cell but are absent in plasma-borne apoB100 or apoB48 (Davis *et al.* 1984, Sparks *et al.* 1987). Acylation may affect the ability of proteins to cross membranes, may assist in protein folding, or facilitate lipid - apolipoprotein association during lipoprotein assembly (Gibbons 1990). Phosphorylation may serve to control assembly, intracellular trafficking and secretion or degradation of apoB100-containing lipoproteins (Davis *et al.* 1984, Sparks *et al.* 1987).

I.3.2. Synthesis and Secretion of ApoB100

Proteins, including apoB100, destined for secretion follow a distinct pathway through a cell. Basically a gene is transcribed and processed into mature mRNA which is then shuttled to the cytoplasm. In the cytoplasm, the

transcript binds to a ribosome which initiates translation. As the nascent polypeptide emerges from the ribosome, the signal recognition particle (SRP) binds to the amino-terminal signal sequence of the peptide. The SRP-ribosome complex is then targeted to the endoplasmic reticulum (ER) to bind the membrane-bound SRP receptor or docking protein. Contact between SRP and its receptor results in dissociation of SRP from the signal sequence followed by the insertion of the signal sequence into the proteinaceous hydrophilic channel (translocon). The nascent peptide is translocated through the channel as translation proceeds. The peptide is therefore sequestered into the lumen of the ER co-translationally. The signal peptide is cleaved as translation proceeds and the peptide is folded and modified before export by vesicle to the Golgi for further modification and eventual secretion from the cell (for a complete review on protein translocation and the secretory pathway refer to Gilmore 1993, Reithmeier 1991).

I.3.2.1. Gene Expression

The apoB gene spans 43 kb and gives rise to a 14,121 nucleotide transcript (Figure I.3). The coding region of the gene contains 29 exons and 28 introns (Blackhart *et al.* 1986). The first 25 exons encode for the amino-terminal third of apoB100 while the carboxyl-terminal two-thirds of the protein are encoded by the remaining four exons, the largest of which are exons 26 and 29 (7572 and 1906 nucleotides in length, respectively) (Blackhart *et al.* 1986).

The mature apoB transcript consists of a 5' untranslated region of 128 nucleotides, a coding region of 13,689 nucleotides (4563 amino acid residues), a UAA stop codon and a 3' untranslated region of 301 nucleotides. A single transcription start site has been demonstrated by primer extension analysis (Protter *et al.* 1986). A canonical polyadenylation signal (AAUAAA) is found 22 nucleotides upstream of the polyadenylation site (Scott 1990). A second cryptic polyadenylation signal (AUUAAA; nucleotide position 6752 of the apoB cDNA) on the edited apoB message may be recognized in the intestine to result in the formation of a transcript of ~7 kb in length (shown in Figure I.3; Chen *et al.* 1987, Powell *et al.* 1987; editing is discussed in section I.4.).

Analysis of genomic DNA in the 5' flanking region has revealed two classic promoter elements - a TATA box 29 nucleotides upstream from the transcription start site and a CAAT box 31 nucleotides upstream of the TATA box. Transcription of the apoB gene transcription is tightly regulated, involving complex interactions between multiple *trans*-acting regulatory proteins with *cis*-acting DNA sequences found in 5' flanking sequences and within introns (Table I.3). For example, the proximal region (-86 to -70) contains a number of response elements known to bind a variety of factors including apolipoprotein factor-1 (AF-1). AF-1 (120 - 200 kD) is probably the same protein as liver and intestine transcription factor-1 or liver specific transcription factor-A1 (LIT-1/LF-A1, respectively; Zannis *et al.* 1993). Nuclear factor-BA1 (NF-BA1), a protein of 60 kD, and CCAAT/enhancer binding protein (C/EBP), a 42 kD protein which contains an amino-terminal

DNA binding basic region followed by a carboxyl-terminal "leucine zipper" motif typical of a number of transcription factors/activators, also bind to the proximal region. Many of the binding proteins are expressed primarily in the liver or intestine and their response elements are common to other genes expressed in these tissues including apoAI, apoAII and apoCIII (Zannis *et al.* 1993, Scott 1990). The proximal promoter region also contains sites recognized by members of the steroid hormone receptor superfamily: ApoAI response protein-1(ARP-1), v-erbA-related human receptor-2 and 3 (EAR-2 and EAR-3, respectively), and hepatic nuclear factor-4 (HNF-4) (Ladias *et al.* 1992). ARP-1, EAR-2 and EAR-3 are negative regulators of transcription and may compete with HNF-4 for sequence binding, whereas HNF-4 may work synergistically with C/EBP to activate transcription (Metzger *et al.* 1993). Another ARP-1 binding site has been localized to within nucleotides -2801 to -2778 from the transcription start site and may reduce transcriptional activity in a tissue specific manner (Paulweber *et al.* 1991c and 1993). Notably, the ligands for ARP-1, EAR-2, EAR-3 and HNF-4 have not been isolated. It has been suggested that these factors may bind ligand(s) that is(are) an intermediate(s) in lipid metabolism or signal transduction such that apoB gene transcription is regulated by the metabolic state of the cell (see section I.3.3.) (Metzger *et al.* 1993, Sparks and Sparks 1993).

I.3.2.2. Synthesis of ApoB100 Protein and Translocation into the ER Lumen

Like other secreted proteins, apoB100 is synthesized on attached ribosomes of the endoplasmic reticulum. The typical signal peptide of 27

amino acids is cleaved co-translationally. As indicated earlier, apoB100 has no complete transmembrane sequences therefore translocation should proceed without interference. However, apoB100 may differ from other secretory proteins in one respect - it possesses sequences that confer a delay or pause in the translocation process (Chuck *et al.* 1990, Chuck and Lingappa 1992, Chuck *et al.* 1993) although this phenomenon has not been confirmed by other investigators (Pease *et al.* 1991 and 1995). It is speculated that pauses in apoB100 translocation may enable the nascent peptide to interact with resident ER proteins such as chaperones which facilitate protein folding, or allow the peptide to associate with lipid.

Cell specific protein factors may also be important for translocation of apoB100 as suggested by transfection studies. For example, apoB53 (representing 53 % of the amino-terminal portion of apoB100) expressed by CHO (Chinese hamster ovary) cells was not secreted and was only partially translocated, whereas the majority of apoB15 (representing 15 % of the amino-terminal portion of apoB100) produced by these cells was translocated and secreted (Thrift *et al.* 1992). In addition, transfected hepatoma cell lines were able to assemble and secrete apoB100 truncation variants larger than apoB28 (representing the amino-terminal 28 % of apoB100) while transfected COS-7 cells (a monkey kidney cell line) could not (Yao *et al.* 1991). Indeed, a factor has been discovered that confers the ability to assemble and secrete lipoproteins in cells that do not normally express apoB100 (Gordon *et al.* 1994, Leiper *et al.* 1994). The factor, microsomal triglyceride transfer protein (MTP), is an important factor for the

assembly of apoB-containing lipoproteins. MTP is a heterodimer consisting of a 97 kD subunit which catalyses the transport of triglycerides, cholesteryl esters and phospholipids between phospholipid surfaces, and a 58 kD subunit, protein disulfide isomerase (PDI), a multifunctional protein, one of its functions of which may be to catalyze the isomerization of intramolecular disulfide bonds during protein folding in the ER (Wetterau and Zilversmit 1985, Wetterau *et al.* 1991, Noiva and Lennarz 1992). While PDI is expressed ubiquitously, the 97 kD subunit has only been detected in the liver and intestine (Wetterau *et al.* 1992). In patients with abetalipoproteinemia, a disease in which the assembly and secretion of apoB100-containing lipoproteins is defective, MTP is absent (Wetterau *et al.* 1992, see section I.5.).

I.3.2.3. Post-translational Degradation

It appears that much of the apoB100 protein that is synthesized is degraded within the cell. The subcellular location of apoB degradation has been the object of intense study. Davis *et al.* (1989) demonstrated that proteolytic fragments derived from apoB100 were detectable in the ER but were absent from the Golgi. A later study by the same group suggested that there were two populations of apoB100: a pool that remained cytosolically exposed and was subsequently degraded and a second pool which was translocated into the ER lumen and subsequently utilized for lipoprotein assembly (Davis *et al.* 1990). Alternately, or in addition to cytosolic degradation, degradation could occur within the ER as suggested by Sato *et al.* (1990) and Furukawa *et al.* (1992). Although the protease(s) involved is

unknown, the enzyme(s) may be a thiol protease since apoB100 is protected from degradation in the presence of thiol protease inhibitors (*e. g.*, N-acetyl-leucinyl-leucinyl-norleucinal, chymostatin) (Sparks and Sparks 1993; references cited therein). In addition, apoB100 may possess sequences that specify its degradation (*e. g.*, sequences similar to other proteins that undergo degradation such as the T cell receptor alpha subunit, or PEST sequences such as those found in cyclins which are rapidly degraded on completion of the cell cycle) (Davis *et al.* 1990, Sparks and Sparks 1993).

I.3.2.4. Current Model of ApoB100 Lipoprotein Assembly

It is currently thought that the lipid constituents of an apoB100-containing lipoprotein associate with the growing apoB100 peptide chain as it enters the ER lumen (co-translationally) via the activity of MTP, chaperones, and lipid synthesizing enzymes (*e. g.*, diacylglyceryl acyl transferase). As determined by puromycin release studies, the nascent lipoproteins achieve a size and density reliant on the amount of apoB100 translated (Spring *et al.* 1992b, Borén *et al.* 1992). Further addition of lipid may occur at the junction of the rough and smooth ER via a so-called "second step" (Alexander *et al.* 1976). In this process, a lipid droplet fuses with the fully translocated apoB100-containing particle (Schumaker *et al.* 1994). Conversely, the nascent particle may be degraded within the ER if insufficiently lipidated. The newly-assembled lipoproteins may then undergo further remodeling and modification in post-ER compartments before eventual secretion.

### I.3.3. Regulation of ApoB100 Synthesis and Secretion

#### I.3.3.1. Regulation of ApoB Gene Expression

Evidence from a number of studies (Pullinger *et al.* 1989, Moberly *et al.* 1990, Dashti *et al.* 1989, Sorci-Thomas *et al.* 1989, Kushwaha *et al.* 1991) demonstrate that the steady state level of apoB mRNA is tightly controlled such that apoB mRNA remains relatively constant under conditions where the level of apoB100 secretion changes by several fold. However, evidence from *in vitro* studies suggests that the steady state level of apoB mRNA can change albeit the magnitude of the change is small (less than 2-fold). For example, an increase in apoB mRNA levels (55 % increase over control) was observed on the addition of 25-hydroxycholesterol to the human hepatoma cell line, HepG2, while free cholesterol had no effect (Dashti 1992). Increases in apoB mRNA have also been observed in HepG2 cells treated with media supplemented with low concentrations of amino acids (0.5 mg/ml) as opposed to high concentrations of amino acids (5.5 mg/ml) (87±22 % increase, low *versus* high) (Zhang *et al.* 1993). In a recent report, a 20 - 30 % increase in apoB mRNA was observed in HepG2 cells on supplementation with VLDL (Wu *et al.* 1994). In all cases, secretion of apoB100 by HepG2 cells was increased, suggesting that the level of apoB mRNA positively affects apoB100 secretion. Altered apoB mRNA concentrations were also observed in *in vivo* studies. For instance, cebus monkeys fed a high cholesterol, coconut oil diet for 3 years also demonstrated elevated apoB mRNA (87 % greater levels) than monkeys fed corn oil (Hennessy *et al.* 1992). Elevated apoB mRNA levels were also

found in liver biopsies from diabetic rats given insulin for 7 days (233±19 % over control) (Sparks *et al.* 1992). Furthermore, increased levels of apoB mRNA and intracellular protein were found in liver biopsies from patients with abetalipoproteinemia (Lackner *et al.* 1986, see section I.5.1.). Conversely, reduced levels of apoB mRNA have been observed in liver biopsies from some patients homozygous for familial hypobetalipoproteinemia, an autosomal codominant disorder associated with truncated forms of apoB100 (Ross *et al.* 1987; see section I.5.2.).

In sum, even though many results suggest that the level of apoB gene transcription or the level of apoB mRNA may not be responsive to changes in lipid metabolism, evidence does exist that expression of the apoB gene can be altered. One of the objectives of this thesis is to investigate the relationship between apoB mRNA level and secretion of apoB-containing lipoproteins.

I.3.3.2. Regulation of ApoB100 Protein Synthesis and Translocation

Changes in metabolic status may affect apoB100 production at the level of protein synthesis. For example, the rate at which apoB100 was synthesized in rat hepatocytes was reduced upon the addition of insulin (Sparks and Sparks 1990). Zhang *et al.* (1993) also observed that the initial amount of apoB100 synthesized was enhanced in HepG2 cells (10.5±3.8 X $10^3$ *versus* 5.7±1.7 X $10^3$ dpm [$^3$H] leucine incorporated into apoB100/mg cell protein) grown in media supplemented with low as opposed to high amounts of amino acids.

The phospholipid content of the ER membrane may greatly influence the ability of the nascent apoB100 peptide to translocate into the ER lumen. For instance, secretion of apoB100-containing lipoproteins is impaired in hepatocytes of rats fed monomethylethanolamine (Vance 1991). Feeding rats this precursor resulted in the accumulation of phosphatidylmonomethylethanolamine (PMME) in the ER membrane. PMME was found to specifically inhibit translocation of apoB100 since translocation and secretion of albumin was not affected (Vance 1991, Rusiñol *et al.* 1993). As a consequence, the number of VLDL particles secreted from the PMME-enriched liver was reduced (Vance 1991, Rusiñol *et al.* 1993).

I.3.3.3. Post-translational Regulation

The first indication that regulation of apoB100 secretion occurs primarily at the post-translational level came from the studies of Borchardt and Davis (1987) who reported that only 36 and 60 % of the newly synthesized apoB100 and apoB48, respectively, were secreted by cultured hepatocytes. Boström *et al.* (1988) found that the inclusion of oleate in the medium of HepG2 cells increased the proportion of the apoB100 pool that was secreted. Furthermore, Dixon *et al.* (1991) observed that oleate could modulate the extent of apoB100 degradation: in the presence of oleate, apoB100 degradation was reduced and its secretion enhanced. In contrast, treatment of hepatocytes with insulin not only decreased total apoB100 synthesis but also stimulated intracellular degradation of newly synthesized apoB100 (Sparks and Sparks 1990).

Effects of hormones and lipid substrates on apoB100-containing lipoprotein production are summarized in Table I.4.

## I.4. APOLIPOPROTEIN B48

Structurally, apoB48 is colinear with the amino-terminal 48 % of apoB100, as determined by protein sequencing, peptide mapping, and mapping epitope positions of monoclonal antibodies (Innerarity *et al.* 1987, Marcel *et al.* 1987). Both apoB48 and apoB100 are products of the same apoB gene and a single transcript. However, the transcript of apoB48 differs from the apoB100 transcript in one respect: a single uridine (U) for cytidine (C) substitution at nucleotide 6666. This change, refered to as RNA editing, results in the conversion of a glutamine codon (CAA) to an in frame stop codon (UAA). Thus, translation of the edited mRNA produces a protein of 2152 amino acid residues instead of the full length 4536 residues of apoB100 (Powell *et al.* 1987, Chen *et al.* 1987).

ApoB mRNA editing is a post-transcriptional event that occurs in the nucleus coincident with RNA splicing and polyadenylation (Lau *et al.*, 1991). The process must be very specific given that there are 3135 cytidines in mature apoB mRNA of which primarily a single cytidine is edited. Indeed, the process is thought to depend on a minimum of 26 nucleotides encompassing the edited cytidine (nucleotides 6662 - 6687) (Shah *et al.* 1991). Mutagenesis studies indicate that the sequence requirements within the region immediate to the edited cytidine are lax (Chen *et al.* 1990, Yao *et al.* 1992). However, a 3' 11-base flanking sequence that must be 5

nucleotides downstream of the edited cytidine is essential for specificity (the mooring sequence: nucleotides 6671 - 6689) while the efficiency of editing can be enhanced by additional sequences 5' of the editing site (regulator sequences) (Chen *et al.* 1990, Smith *et al.* 1991, Backus and Smith 1992). It has also been suggested that the overall secondary structure of apoB mRNA may dictate the position of editing (Smith, 1993).

The C to U editing reaction involves deamination of the cytidine (Boström *et al.* 1990, Hodges *et al.* 1991, Johnson *et al.* 1993). Teng *et al.*(1993) cloned the cDNA for a protein of 27 kD (p27) from the rat intestinal cDNA library. When the p27 cDNA was expressed in *Xenopus* extracts, it could enhance editing activity *in vitro*. Based on the predicted amino acid sequence, the protein was found to share considerable homology to the active site of the cytidine deaminase of *Escherichia coli*, *Bacillus subtillis*, yeast and human, as well as deoxycytidylate deaminases of T2 and T4 bacteriophage and humans (Navaratnam *et al.* 1993a). The p27 protein also demonstrates deaminase activity *in vitro* (Navaratnam *et al.* 1993a). Notably, p27 is inactive in *in vitro* editing assays unless the system is supplemented with intestinal extract, suggesting other factors are important for its function (Teng *et al.* 1993, Navaratnam *et al.* 1993a). Indeed, proteins of 60 kD and 40 kD have been crosslinked to apoB mRNA substrates (Lau *et al.* 1990, Harris *et al.* 1993, Navaratnam *et al.* 1993b) The 60 kD protein binds nucleotides 6671 - 6674 and thus may serve as the recognition factor for the editing process (Navaratnam *et al.* 1993b, Harris *et al.* 1993). Smith (1993) proposes that the proteins form an "editosome" complex that

sediments at 27 S. Therefore, a multicomponent complex may be required to ensure accurate and efficient apoB mRNA editing.

ApoB mRNA editing appears to be tissue specific (Teng *et al.* 1990a). In mammals it occurs very efficiently in the small intestine which produces primarily the apoB48 protein. In rats, mice, horses and dogs, editing of the apoB message also occurs in the liver so that both apoB100 and apoB48 are produced (Greeve *et al.* 1993). Editing has also been detected in tissues that do not express apoB100 or apoB48, suggesting that other substrates for editing may exist (Teng *et al.* 1990a, Boström *et al.* 1990). The editing mechanism seems to be conserved in mammals based on interspecies nucleotide homology in apoB cDNA surrounding the editing site (Teng *et al.* 1990b) and based on the ability of mouse transgenics to efficiently edit human apoB mRNA (Chiesa *et al.* 1993, Young *et al.* 1994). Editing activity does not exist in avian species although chicken intestinal extracts possess a stimulatory factor(s) that enhances editing activity of rat intestinal extracts (Teng and Davidson 1992).

ApoB mRNA editing is a novel mechanism of gene regulation and may have significant consequences with respect to lipoprotein metabolism, *i. e.*, apoB48 lacks the LDL receptor binding region present on apoB100 yet apoB48-containing lipoproteins are metabolized very rapidly compared to those containing apoB100 (Chan and Boerwinkle 1992). The editing process is also responsive to hormonal stimuli (*e. g.*, thyroid hormones, insulin) and nutritional status, and is subject to developmental regulation

(Davidson *et al.* 1988, Sparks *et al.* 1992, Thorngate *et al.* 1994, Harris and Smith 1992, Wu *et al.* 1990, Teng *et al.* 1990a and b).

## I.5. DISORDERS OF APOB100 LIPOPROTEIN PRODUCTION

### I.5.1. Abetalipoproteinemia

Abetalipoproteinemia (abeta) is a rare congenital disorder usually transmitted as a recessive trait. It is characterized by fat malabsorption, failure to thrive, ataxia neuropathy, retinitis pigmentosa, and acanthocytosis (Kane and Havel, 1989). In the affected subjects, low levels of plasma cholesterol and triglyceride and almost complete absence of plasma apoB100- and apoB48-containing lipoproteins are observed. Liver biopsies obtained from abeta patients show normal or increased levels of the apoB mRNA and intracellular apoB protein relative to samples from normal livers (Lackner *et al.* 1986). Intestinal organ cultures of biopsies from two patients with abeta also showed normal apoB48 synthesis but an absence of intracellular lipoprotein molecules (Bouma *et al.* 1990). These findings suggest that this condition reflects a defect in apoB100- and apoB48-containing lipoprotein assembly rather than a mutation within the apoB gene. The lack of an involvement of the apoB gene in abeta is also supported by genetic studies - *i. e.*, the defect does not appear to be linked to the apoB locus (Talmud *et al.* 1987, Huang *et al.* 1990). Recently, it has been shown that some patients with abeta lack the 97 kD component of microsomal triglyceride transfer protein (MTP), (Wetterau *et al.* 1992). Furthermore, when the cDNA for the human 97 kD subunit was cloned and

compared to the genomic sequences from two abeta patients, one patient demonstrated a homozygous frameshift mutation while the other a homozygous nonsense mutation, indicating that a defect in the gene for MTP may be the cause of this disease (Sharp *et al.* 1993). Thus the activity of this protein may be important for assembly of apoB100- and apoB48-containing lipoproteins.

### I.5.2. Hypobetalipoproteinemia

Familial hypobetalipoproteinemia (hypobeta) is an autosomal codominant disorder characterized by decreased or absent plasma concentrations of the apoB100-containing lipoproteins (for review see Linton *et al.* 1993a). While obligate heterozygotes for abeta (*i. e.*, the parents of abetalipoproteinemics) have normal plasma lipid and lipoproteins, heterozygotes for hypobeta have plasma concentrations of apoB100 and LDL-cholesterol that are reduced compared to normal (Linton *et al.* 1993a). Patients homozygous for hypobeta lack plasma apoB100 and apoB100-containing lipoproteins and can be phenotypically indistinguishable from patients with abeta. Homozygous abeta patients display normal or elevated intracellular apoB mRNA and protein, whereas homozygous hypobeta patients display decreased levels of intracellular apoB mRNA and protein (Ross *et al.* 1987). In some kindreds hypobeta cosegregates with truncated forms of apoB100. To date, over 20 different apoB truncations have been identified. In general, the short apoB forms have reduced capacity to assemble lipoprotein particles as compared to those that are larger than apoB48.

Recently, Maeda and coworkers (Homanics *et al.* 1993) used gene targeting to interrupt exon 26 of the mouse apoB gene and generated heterozygous and homozygous mice that produced a truncated apoB100 protein. These mice demonstrated phenotypes similar to hypobeta, including reduced apoB mRNA levels in the homozygous state. The mechanism(s) underlying the hypobeta phenotype has(have) not been pursued in this model system. It has been suggested that the abnormally low plasma apoB concentrations in homozygote and heterozygote hypobetalipoproteinemics may arise from decreased apoB production, enhanced clearance, or a combination of both (Young 1990, Linton *et al.* 1993a).

### I.5.3. Hyperapobetalipoproteinemia

Hyperapobetalipoproteinemia (hyperapobeta) refers to a subset of dyslipoproteinemias characterized by VLDL of normal composition, increased secretion rate of apoB100-containing lipoproteins, increased LDL particle number, and escalated risk of premature coronary heart disease. Overproduction of hepatic apoB100 lipoproteins in hyperapobeta may be due to increased delivery of fatty acid or sterol, or both, to the liver (Sniderman and Cianflone 1993). For example, hyperapobeta may be a consequence of reduced uptake of fatty acid by adipose tissue, either by virtue of a deficiency in lipoprotein lipase activity or by lack of responsivity perhaps to acylation stimulating protein (ASP) such that fatty acid is not converted to triglyceride for storage (Babriak *et al.* 1989, Babriak *et al.* 1992, Teng *et al.* 1987, Baido *et al.* 1993). As a consequence, delivery of fatty acid

(free or as chylomicron remnants with a higher triglyceride content than normal) to the liver increases, resulting in increased production of apoB100-containing lipoproteins. It is unknown if an abnormality in apoB gene expression or apoB100-containing lipoprotein production could be a cause of the disorder.

The role of apoB100 in lipoprotein metabolism and atherogenesis has been studied using transgenic mice expressing human apoB100. The transgenic mice demonstrated a positive correlation between human apoB gene copy number and plasma human apoB100 levels in the mice (Linton *et al.* 1993b, Callow *et al.* 1994). Virtually all of the human apoB100 in the transgenic plasma was found in the LDL fraction and resulted in substantially higher LDL- and total plasma- cholesterol (Callow *et al.* 1994, Young *et al.* 1994) and triglyceride levels (Young *et al.* 1994) compared to nontransgenic littermates. The metabolic basis for the accumulation of human apoB100 in the transgenic mice is as yet unknown (Young *et al.* 1994). Recent work by Young and colleagues (Percell-Huynh *et al.* 1995) has demonstrated that mice expressing human apoB in the setting of a diet rich in fat develop severe atherosclerosis.

## I.6. MCARDLE RAT HEPATOMA 7777

While the current understanding of the physiology and metabolism of lipoproteins has been achieved from studies using intact animal or organ perfusion, cell culture has facilitated the investigation of lipoprotein metabolism at the cellular level. Both primary hepatocytes and hepatoma

cell lines have been used to examine the assembly and secretion of lipoproteins under a wide range of conditions. The different cell culture systems, however, have certain advantages and disadvantages. For instance, while primary hepatocytes often reflect the metabolic state of the donor animal (*e. g.*, fasting *versus* fed), they do so for a limited time before they dedifferentiate (being of a mixed cell population, cells of less defined lineage overgrow) and having finite cell division, die. Hepatoma cell lines, in contrast, are easy to obtain, maintain and provide the assurance of long term cell growth and division. Often these cells retain many functions of the cells from which they were derived.

In 1960, Potter, Morris, and colleagues decided that an experimental cancer model would be an invaluable tool to examine neoplastic changes in cells. Since they were studying processes unique to liver tissue, they determined that a transplantable liver cancer model would be the ideal system to work with. Over the next 18 years, Morris undertook a series of rat studies using several chemicals known to cause liver cancer and developed numerous strains of hepatomas. These strains demonstrated a wide variety of phenotypes ranging from poorly differentiated, rapidly growing to very slowly growing, highly differentiated cancer tissues, and a nearly normal complement of enzymes that carry out the functions specific to the liver (Morris and Criss 1978).

In 1972, Narayan and Morris reported production of large quantities of lipoproteins by one of the tumors Morris developed - the solid tumor Morris hepatoma 7777. The solid tumor and primary cultures derived from the

tumor then became the focus of intermediary metabolic studies including lipoprotein metabolism (Grigor *et al.* 1973, Morton *et al.* 1982, Yoo *et al.* 1980, Abbas *et al.* 1982). In 1974, Becker *et al.* (1976) established the tumor as a stable cell line. This cell line was designated McArdle-RH7777 (McA-RH7777) and was found in subsequent studies to retain the lipoprotein-producing phenotype of the parent tumor (Tanabe *et al.* 1989, Yao *et al.* 1993). McA-RH7777 cells express most apolipoproteins and, unlike primary rat hepatocytes, express low levels of apoB48 due to low editing efficiency (Yao *et al.* 1993, Driscoll and Zhang 1994; references cited within). In both model systems, however, apoB48 can be found on HDL sized particles as well as VLDL, albeit to a lesser extent in the rat hepatoma cell line unless the cells are supplemented with oleate (Borén *et al.* 1994, Yao *et al.* 1993; references cited within). ApoB100-containing lipoproteins produced by McA-RH7777 cells are primarily LDL and IDL in size and attain VLDL proportions on supplementation with oleate (Borén *et al.* 1994).

## I.7. THESIS OBJECTIVE

### I.7.1. Effect of Carboxyl-terminal Truncation of ApoB100 on Lipoprotein Production

From the clinical viewpoint, there is much interest in the study of the production of apoB100-containing lipoproteins since elevated levels of plasma apoB100 are strongly correlated to the development of coronary heart disease (Kesaniemi *et al.* 1983; Sniderman *et al.* 1980; Chan and Boerwinkle 1992). Much has been learned by studying the effects of altered

nutritional, hormonal and pharmacological states on apoB100 lipoprotein production, but much more has yet to be learned about the process, and in particular the role that apoB100 plays in the process. Preliminary studies suggested that apoB100 may serve as the structural backbone for assembly of apoB100-containing lipoproteins in the liver, *i. e.*, with increasing length of apoB100 synthesized and translocated into the lumen of the ER, lipoproteins form that exhibit progressive decrease in density and increase in size due to lipidation. However, this relationship had only been established for carboxyl-terminal truncation variants with little lipid core, it is unknown if this relationship holds for larger truncation variants. In addition, while much of apoB100 synthesized is never secreted but is degraded, it is unknown if specific regions of apoB100 could be important in determining the kinetics of secretion. Therefore the intent of the first part of this thesis is to examine the role of the carboxyl terminus of apoB100 in the assembly and secretion of apoB100-containing lipoproteins by the liver. To achieve this goal, McA-RH7777 cell lines stably expressing human apoB100 carboxyl truncation variants apoB64, apoB72 or apoB100 protein (representing the amino-terminal 64-, 72- or 100- % of the full length) were generated and studied. A virtue of this system is that the rat hepatoma provides the basic machinery for lipoprotein assembly while the human apoB variants can be easily detected against the rat background.

### I.7.2. Effect of Overexpression of ApoB53 on Lipoprotein Production

It is unknown whether changes in the levels of apoB mRNA have significant effects on production of apoB100-containing lipoproteins. Some

*in vitro* studies suggest that apoB mRNA levels fluctuate little in the face of a wide variety of stimuli while the amount of protein secreted can be influenced dramatically. However, the conditions utilized in these studies may not have met the physiological conditions required for substantial changes in apoB mRNA levels to occur. Other studies have shown that changes in levels of apoB mRNA can occur, however, the stimuli utilized in these studies may have exerted effects at multiple stages of apoB100-containing lipoprotein production, so that the contribution made by changes in mRNA levels may be difficult to discern. Therefore, the second goal of this thesis is to determine the effects of overexpression of apoB100 on lipoprotein production. Since substantial differences in expression level of full length apoB100 were difficult to achieve, McA-RH7777 cells were used to generate cell lines that in this case express vastly different steady state levels of human apoB53 (representing the amino-terminal 53 % of apoB100). The effects of expressing different levels of apoB53 mRNA on the synthesis, secretion, and composition of the human apoB53-containing lipoproteins were determined.

**Figure I.1:** Schematic representation of apoB100 on an LDL particle (after Yao 1994, unpublished). - represents the phospholipid monolayer surrounding the neutral lipid core (in grey). - represents apoB100.

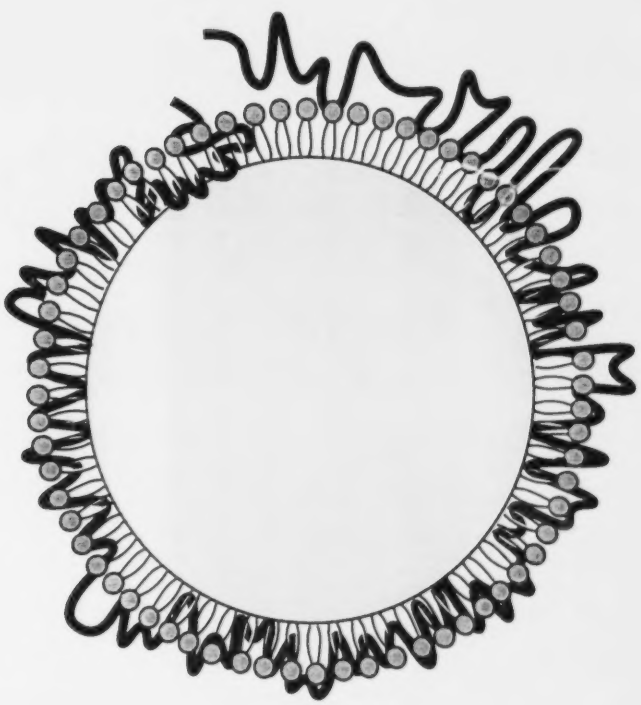
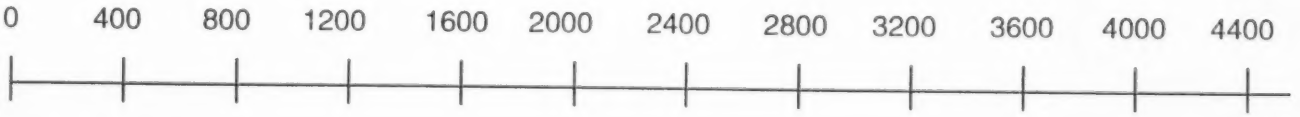


**Figure I.2:** Linear representation of apoB100 indicating distribution of cysteines (A.) and N-glycosylation sites (B.) (after Chan 1992). Each horizontal line corresponds to apoB100 with the amino terminus on the left, the carboxyl terminus on the right and amino acid positions indicated on the scale below A. and B. In A. single vertical lines and brackets represent the positions of free cysteines and disulfide bridges, respectively. A question mark above a vertical line denotes status unknown. In B. vertical lines indicate potential glycosylation sites; asterisks denote the known glycosylation sites.

**A. Distribution of Cysteines**

**B. Distribution of N-glycosylation sites**




*amino acid#*

**Figure I.3:** Organization of the apoB gene and origins of apoB100 and apoB48 transcripts (mRNA) (after Scott 1989). Exons are designated as black lines or bars, introns as thin lines between the exons. The location of the two large exons is indicated. Production of apoB48 is discussed in section I.4.

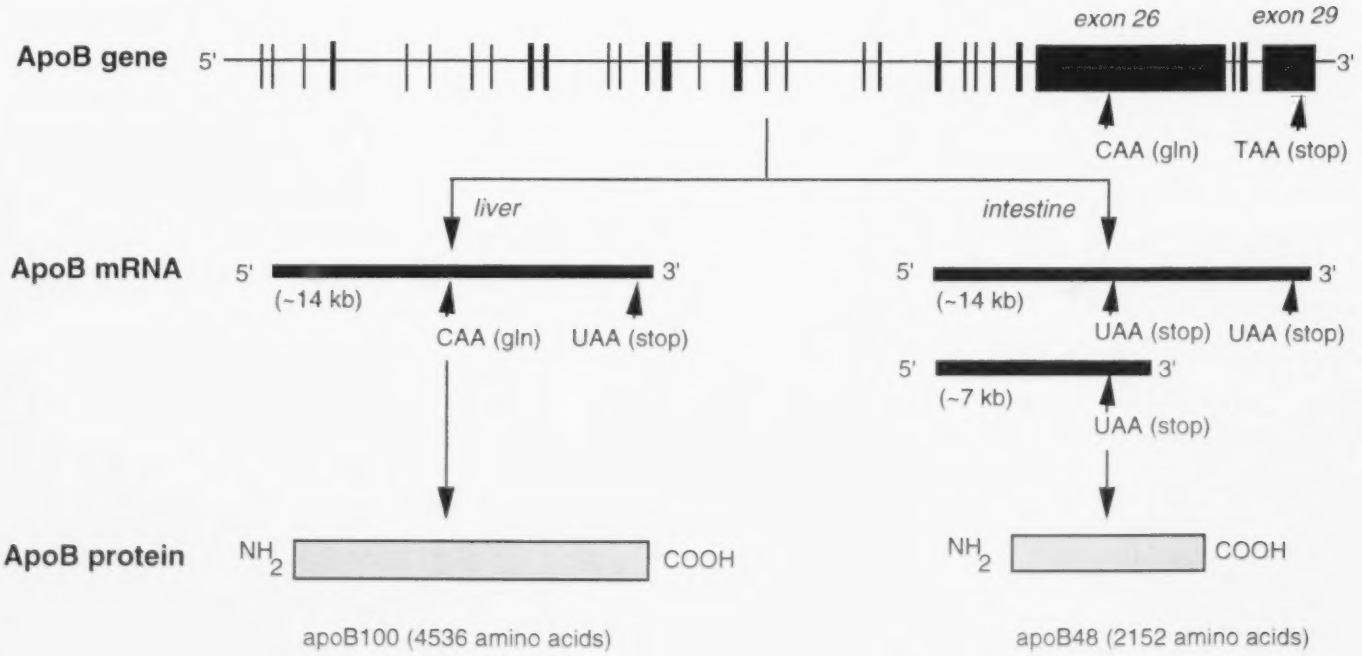


**Table I.1:** Physicochemical properties of human plasma lipoproteins (after Havel and Kane 1989, Zannis *et al.* 1993). (FC, free cholesterol; PL, phospholipid; CE, cholesteryl ester; TG, triglyceride)

| class | density | diameter | molecular mass | composition *(% dry mass)* | | | | |
|---|---|---|---|---|---|---|---|---|
| | *(g/ml)* | *(nm)* | *($X10^6$ D)* | protein | FC | PL | CE | TG |
| chylomicron | 0.93 | 75-1200 | 50-1000 | 2 | 2 | 7 | 3 | 86 |
| VLDL | 0.93-1.006 | 30-80 | 10-80 | 8 | 7 | 18 | 12 | 55 |
| IDL | 1.006-1.02 | 25-35 | 5-10 | 19 | 9 | 19 | 29 | 23 |
| LDL | 1.02-1.063 | 18-25 | 2.3 | 22 | 8 | 22 | 42 | 6 |
| $HDL_2$ | 1.063-1.125 | 9-12 | 0.36 | 40 | 5 | 33 | 17 | 5 |
| $HDL_3$ | 1.125-1.210 | 5-9 | 0.175 | 55 | 4 | 35 | 13 | 3 |

**Table I.2:** Characteristics of plasma apolipoproteins in humans adapted from Havel and Kane 1989, Fielding and Fielding 1991, Zannis *et al.* 1993). (LCAT, lecithin cholesterol acyltransferase; HL, hepatic lipase; LPL, lipoprotein lipase)

| apolipoprotein | plasma concentration *(mg/dl)* | molecular mass *(kD)** | major site of synthesis | chromosomal location | lipoprotein distribution | function |
|---|---|---|---|---|---|---|
| AI | 100-130 | 28.1 (243) | small intestine, liver | 11q | HDL | LCAT activator |
| AII | 30-50 | 17.4 (77) | small intestine, liver | 1q | HDL | LCAT activator |
| AIV | 15 | 43.0 (376) | small intestine | 11q | HDL | LCAT activator |
| B100 | 80-120 | 550.0 (4536) | liver | 2p | VLDL, IDL, LDL | VLDL assembly, LDL receptor ligand |
| B48 | <5 | 250.0 (2152) | small intestine | 2p | chylomicron | chylomicron assembly |
| CI | 5-7 | 6.6 (57) | liver | 19q | chylomicron, VLDL, HDL | LCAT activator (moderate), inhibits catabolism of apoE containing lipoproteins |
| CII | 3-7 | 8.0 (79) | liver | 19q | chylomicron, VLDL, HDL | LPL activator |
| CIII | 9-13 | 8.75 (79) | liver | 11q | chylomicron, VLDL, HDL | inhibits catabolism of apoE-containing lipoproteins |
| D | 6-7 | 33.0 (169) | adrenal, kidney, brain, liver, small intestine | 3q | HDL | cholesteryl ester exchange |
| E | 3-6 | 34.2 (299) | liver, macrophage, astrocytes in brain | 19q | chylomicron remnant, VLDL, HDL | ligand of LDL receptor and putative remnant receptor |

* number of amino acids of the mature proteins are shown in parentheses

**Table I.3:** *Cis-* and *trans*-acting factors involved in apoB gene regulation (compiled from Zannis *et al.* 1993, Paulweber *et al.* 1991a, b, c, and 1993, Ladias *et al.* 1992, Metzger *et al.* 1993, Brooks *et al.* 1994). (+, activates transcription; -, inhibits transcription)

| regulatory region | genomic location *(relative to transcription start site)* | binding factors | effect of binding *(activate or inhibit transcription)* | comment |
|---|---|---|---|---|
| proximal promoter | -86 to -70 | AF-1 | + | required for expression or suppression in intestinal and liver cells |
| | | C/EBP | + | |
| | | HNF-4 | + | |
| | | ARP-1 | - | |
| | | EAR-2 | - | |
| | | EAR-3 | - | |
| distal promoter | -128 to -86 | BRF-2 | + | required for expression in HeLa (cervical carcinoma) cells |
| enhancer | 2nd & 3rd intron | HNF-1 | + | required for liver specific expression in transgenic mice |
| | | HNF-3 | | |
| | | C/EBP protein II | | |
| reducer | -2000 to -3000 | ARP-1 | - | exerts effect on intestinal cells; no effect on liver cells |
| | | EAR-2 | - | |
| | | EAR-3 | - | |

**Table I.4**: Factors affecting production of apoB-containing lipoproteins from the liver. (ND - not determined)

| factor | effect | | | system | reference |
|---|---|---|---|---|---|
| | mRNA synthesis | protein synthesis /translocation into ER | secretion into media /plasma | | |
| *hormone:* | | | | | |
| insulin (acute) | no effect | decrease synthesis | decrease | rat hepatocyte | Sparks and Sparks 1990 |
| dexamethasone | ND | ND | increase | rat hepatocyte | Martin-Sanz*et al.* 1990 |
| glucagon/cAMP | ND | ND | decrease | rat hepatocyte | Björnsson *et al.* 1994 |
| cytokines (TNFα, IL-1, IL-6) | no effect | ND | decrease | HepG2 | Ettinger *et al.* 1994 |
| *lipid availability:* | | | | | |
| *i. TG synthesis* | | | | | |
| oleic acid | no effect | no effect | increase | HepG2/rat liver (fasted) | Dixon and Ginsberg 1993 |
| ω-3 fatty acid | no effect | no effect | decrease | rat hepatocyte/ McARH7777* | Wang *et al.* 1993/ Wang *et al.* 1994 |
| carbohydrate | ND | ND | increase particle size** | HepG2 | Cianflone *et al.* 1992 |

**Table I.4:** continued

| factor | effect | | | system | reference |
|---|---|---|---|---|---|
| | mRNA synthesis | protein synthesis /translocation into ER | secretion into media /plasma | | |
| i. TG synthesis cont'd | | | | | |
| fatty acid synthesis inhibitors | no effect/ND | ND/decrease synthesis | decrease/decrease | hamster hepatocyte/ HepG2 | Arbeeny *et al.* 1992/ Wu *et al.* 1994a |
| VLDL | increase | increase synthesis | increase | HepG2 | Wu *et al.* 1994b |
| ii. FC/CE synthesis: | | | | | |
| OHcholesterol | increase | increase synthesis | increase | HepG2 | Dashti 1992 |
| LDL | no effect | increase synthesis | increase | HepG2 | Dashti 1992 |
| HMGCoA reductase inhibitors | ND/no effect | ND/ND | decrease/decrease | HepG2/rabbit hepatocyte | Cianflone *et al.* 1990/ Tanaka *et al.* 1993 |
| ACAT inhibitors | ND/ND | ND/ND | decrease/decrease | HepG2/miniature pig | Cianflone *et al.* 1990/ Huff *et al.* 1994 |
| iii. PL synthesis: | | | | | |
| choline deprivation | ND | no effect | decrease*** | rat hepatocyte | Verkade *et al.* 1993 |
| PMME | ND | inhibits translocation | decrease | rat hepatocyte | Rusiñol *et al.* 1993 |

*rat hepatoma cells expressing human apoB18, B28 and B42
***i. e.*, no change in number of particles secreted as per other stimuli
***inhibition of transport from ER to Golgi observed (*i. e.*, fewer particles found in choline deficient rat liver cell Golgi)

# Chapter II MATERIALS AND METHODS

## II.1. MATERIALS

### II.1.1. Cell Lines, growth media, antibiotics

McA-RH7777 cells were obtained from the American Type Tissue Culture Collection. Culture media, sera, trypsin-EDTA (0.25 % trypsin (w/v), 1 mM ethylenediaminetetraacetate), penicillin G, streptomycin, and neomycin analog, G418, were obtained from Life Sciences, Inc.

### II.1.2. Antibodies

Monoclonal antibody 1D1, 3F5, and 4G3 were gifts from R. W. Milne and Y. L. Marcel (University of Ottawa Heart Institute, Ottawa, ON). Polyclonal antibodies specific for rat apoB, human apoB100 peptide 2140 and human apoAI and apoE were gifts from R. Davis (San Diego State University, San Diego, CA), S. G. Young and T. L. Innerarity (Gladstone Institute of Cardiovascular Disease, San Francisco, CA), and T. Ohnishi and S. Yokoyama (University of Alberta, Edmonton, AB), respectively. Peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G antibodies were obtained from Amersham. Sheep anti-human apoB100 and apoAI antisera were obtained from Boehringer Mannheim.

### II.1.3. Chemicals and reagents

Reagents for polyacrylamide gel electrophoresis and nitrocellulose membranes for Western blotting were obtained from Bio-Rad. The ECL

Western blotting detection system and Rainbow$^{TM}$ molecular weight size marker were purchased from Amersham. Protein A-Sepharose CL-4B beads were obtained from Pharmacia-LKB Biotechnology, Inc. Lipid assay kits (Triglyceride G, Phospholipid B, Cholesterol CII) were obtained from Wako Pure Chemical Industries, Ltd. Paragon agarose gels were purchased from Beckman Instruments. Other reagents were purchased from Sigma, Fisher or BDH.

II.1.4. Buffers and solutions

All solutions were prepared from analytical or assured grade reagents in double distilled deionized water.

II.1.5. Radioactive materials and scintillation fluors

[$^{35}$S]methionine (Tran$^{35}$S-label, 1100 Ci/mmol) and Ecolite scintillation cocktail were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). [$^{3}$H]glycerol (1(3)-$^{3}$H glycerol, 2.6 Ci/mmol) was obtained from Amersham. Hionic fluor was purchased from Canberra-Packard.

II.2. METHODS

II.2.1. Human carboxyl truncated apoB100 expression plasmids

Construction of the expression plasmids encoding human apoB53, apoB72 and full length apoB100 has been published elsewhere (Blackhart *et. al.* 1990, Yao *et al.* 1991, Yao *et al.* 1992, McLeod *et al.* 1994).

Schematic representations of the plasmid constructs and proteins they encode are shown in Figure II.1 and II.2, respectively.

The expression plasmid pB53 LL (LL refers to "Leu-Leu"; see below) was derived from the parental pB53 (Yao *et al.* 1992) which was constructed in the pCMV5 vector using cDNA fragments encoding the amino-terminal 53 % of the human apoB100 protein. The pCMV5 vector was employed since it contains the strong, constitutive human cytomegalovirus immediate early promoter capable of driving the expression of the insert in a wide variety of eukaryotic cells (Andersson *et al.* 1987, Blackhart *et al.* 1990, Sambrook *et al.* 1989). The expression plasmid pB53 LL was constructed in order to eliminate the formation of apoB48 by mRNA editing. The "Leu-Leu" (LL) mutation involved changing codon 2153 from the trinucleotide encoding glutamine (CAA) to that of leucine (CUA) such that when C to U editing occurs (see section I.4. of Introduction), another leucine codon is generated (UUA) rather than a stop codon (UAA) (Yao *et al.* 1992).

The expression plasmids pB64 LL, pB72 LL and pB100 LL (encoding the amino-terminal 64 %, 72 % and entire apoB100 protein, respectively) were prepared from pB53 LL and p3'B, a plasmid containing the last four exons and three introns of the apoB100 gene as well as sequences extending ~3.6 kb 3' of the gene. The pB72 LL and pB100 LL constructs were validated by transient transfection in COS-7 cells and immunoblot analyses (McLeod *et al.*, 1994).

II.2.2. Cell culture and transfection

McA-RH7777 cell lines stably expressing human apoB53 used in this study were prepared as described (Yao *et al.* 1992). McA-RH7777 expressing human apoB64, apoB72 and apoB100 were prepared by Yang Zhao, technician to Dr. Yao (University of Alberta). Cells expressing apoB64 were also prepared by me.

II.2.2.1. Media and cell culture conditions

McA-RH7777 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing penicillin G (100 U/ml medium), streptomycin (100 mg/ml medium), 10 % heat inactivated fetal bovine serum (v/v) and 10 % heat inactivated horse serum (v/v). Stably transfected McA-RH7777 cell lines (see below) were maintained in medium supplemented with 200 mg active G418/ml medium. All cell lines were maintained in 100 mm tissue culture dishes (Falcon) in a humidified atmosphere of 7 % $CO_2$, 37 $^{o}C$. Cells were passaged once every 6 days when 80 % confluent (representing ~2.4 - 3.0 mg total cell protein per dish as determined by the Lowry assay or 2.4 X $10^6$ - 3 X $10^6$ cells per dish as determined by counting with a hemacytometer). McA-RH7777 cells and the stable lines derived from them detach from the substratum if left to grow to greater confluency. Notably, stable cell lines tended to be more strongly adherent to the substratum prior to this stage so that longer (2 - 3 minutes) or a second exposure to the cell dissociating agent trypsin-EDTA was required. After dissociation, cells were thoroughly resuspended in 6 ml of medium and 0.25 ml (representing a

dilution of 1:24 for a maintenance dish) transferred to a 100 mm dish containing 15 ml of fresh medium and the recipient dish agitated to distribute the cells.

Frozen stocks of cells were prepared by combining trypsinized cells with freezing medium which consisted of 20 % fetal bovine serum (v/v), 10 % of the cryoprotectant dimethyl sulfoxide (v/v) and 70 % DMEM (v/v). Aliquots of the cells to be frozen were then placed in cryovials (Nalge), gradually brought down to -70 $^{o}$C by first incubating the cells on ice for 15 minutes, then transferring them to an insulated box for incubation first at -20 $^{o}$C for 20 minutes followed by overnight at -70 $^{o}$C, and then transferred to liquid nitrogen for storage. Frozen stocks were thawed rapidly in a beaker of Milli-Q water (37 $^{o}$C) and transferred to 15 ml of medium in a 100 mm dish. After incubating overnight, the medium was removed and replaced with fresh medium in order to remove dead cells.

II.2.2.2. Generation of stable transformants

II.2.2.2.1. Preparation of the transfection mixture

The apoB expression plasmids described above were cotransfected with the plasmid pSV2neo into McA-RH7777 cells and selected for G418 resistance (Chen and Okayama 1988). The plasmid pSV2neo contains components of the simian virus SV40 including a promiscuous early gene promoter, as well as the gene for neomycin resistance (neo$^{r}$), aminoglycoside phosphotransferase (Davis *et al.* 1994; references cited therein). Aminoglycoside phosphotransferase inactivates the protein

synthesis inhibitor neomycin and its analogs such as G418. Expression of this enzyme serves as a suitable selectable marker to identify stable integration of exogenous DNA into the genome of the target cell (Davis *et al.* 1994). Previous work had established that cotransfection with two separate plasmids encoding separate genes appears to be as efficient as placing the genes in tandem (reviewed in Sambrook *et al.* 1989).

The transfection mixture was prepared as follows: 5 - 10 µg DNA and 0.3 µg pSV2neo (molar ratio 20:1) were made up to 100 µl with sterile water in a 15-ml sterile capped tube (Falcon) and mixed with 400 µl of 50 mM N,N-bis-(hydroxy-ethyl)-2-amino-ethanesulfonic acid (BES) pH 6.95, 280 mM NaCl, 1.5 mM $Na_2HPO_4$ (BES buffered saline or BBS). The slightly acidic condition (pH 6.95) maintained by the BES component of the buffer allows gradual formation of calcium-DNA precipitates which appears to be critical for efficient stable transformation to occur (Chen and Okayama 1988). The tube was then vortexed while 400 µl 0.315 M $CaCl_2$ was added in dropwise fashion to the tube. The tube was then capped tightly and let stand at room temperature for 20 - 30 minutes. The transfection mixture was vortexed again for 30 seconds just before addition to the cells to be transfected. A vector that had successfully transformed McA-RH7777 cells previously (pB42 LII - encodes for human apoB42 protein) was used as a positive transfection control while 100 µl sterile water without DNA served as the negative control.

II.2.2.2.2. Transfection of cells

McA-RH7777 cells were employed because of their hepatic origin (*i. e.*, the cells normally secrete apoB100- and apoB48-containing lipoproteins) and because they have a transfection efficiency superior to other cell lines (Yao *et al.* 1991). Twenty-four hours before transfection, nearly confluent (60 - 70 % confluent) McA-RH7777 cells were split 1:6 into 75 $cm^2$ canted neck flasks (Falcon). Each flask was then given the transfection mixture. Four hours later, the cells were subjected to glycerol shock. Although the exact mechanism is unknown, osmotic shock induced by the rapid addition and removal of glycerol was found to enhance the efficiency of transfection (Parker and Stark 1979). Basically, this procedure involved aspirating the medium from the cells, adding 3 ml of 15 % glycerol (v/v) in 136.89 mM NaCl, 2.68 mM KCl, 8.11 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, pH 7.4 (phosphate buffered saline or PBS) and letting the solution gently roll over the cells 3 - 4 times. The glycerol was then removed and the cells washed twice with 10 ml sterile PBS. Fresh medium was then added to the cells and the cells returned to the incubator. Two days after the transfection, culture medium was collected to screen for secretion of the recombinant apoB proteins (see section II.2.3.1.1.) and replaced with medium containing G418 (400 μg/ml) to select for stable transformants (selection medium). During the first 7 - 10 days most of the cells died and the selection medium had to be changed every 2 - 3 days. Foci of stably transfected cells formed between 14 - 21 days. These colonies were "picked up" by aspirating most of the selection medium (0.5 ml is retained to keep the cells from drying out) and

adding 5 μl of trypsin-EDTA directly on top of each colony. The colonies were then transferred to individual wells of a 6 well dish (Becton-Dickson) and left for 2 - 3 minutes to be dissociated by the action of trypsin. The cells were then thoroughly mixed to completely dissociate the cells followed by the addition of 4 ml of the selection medium. Once the cells in the 6 well dishes were nearly confluent, the medium was analyzed for expression of the human apoB construct (see section II.2.3.1.1.). Cell lines positive for the expression of human apoB protein were maintained as described (section II.2.2.1.).

II.2.3. Characterization of lipoproteins produced by stably transfected cells lines expressing recombinant human apoB species

II.2.3.1. Gel electrophoresis of cell and media lipoproteins

II.2.3.1.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Samples to be resolved by SDS PAGE were prepared as follows: The conditioned media from transfected cells were collected and centrifuged at 2500 rpm for 5 minutes to remove any cell debris. Typically, a volume of 50 ml of the Cab-O-Sil/water or PBS colloid (50 mg Cab-O-Sil/ml solvent) was added for every 2 ml of the medium and the mixture incubated on ice for 15 minutes with vortexing for 10 seconds at 5 minute intervals (Vance *et al.* 1984, Yao and Vance 1988). The fumed silica-lipoprotein complexes were then sedimented by centrifugation and the pellet rinsed twice to remove the media. Apolipoproteins were then recovered from the Cab-O-Sil with a

protein sample buffer consisting of 6 M urea, 2 % SDS (w/v), 0.65 mM dithiothreitol (DTT), 0.05 % glutathione (w/v), 1.3 % e-aminocaproic acid (w/v), and 0.005 % (w/v) bromophenol blue (BPB) (Lee *et al.* 1981). The samples were heated 10 - 15 minutes at 75 $^{o}$C to denature the proteins and loaded onto SDS polyacrylamide gels.

SDS PAGE was performed using a Hoefer (20 X 20 cm) slab gel apparatus by the method of Laemmli (1970). For resolution of apoB species, a resolving gel of 5 % acrylamide (30 ml) was prepared and overlaid with a stacking gel of 3 % acrylamide (10 ml). A constant current of 8 mA was applied for 16 hours to resolve the protein samples. A lane of 5 - 10 µl Rainbow marker$^{TM}$, a mixture of pre-stained molecular weight standards, was included to estimate molecular weight and allow visual inspection of the completeness of protein transfer if immunoblotting is to be performed (see section II.2.3.2.).

For detection of rat apoAI resolving gels of either 11 % acrylamide or a gradient of 3 - 20 % acrylamide using a gradient former (Bio-Rad) were prepared and run as above. The latter gel also permitted resolution and detection of apoB species (human and rat) as well as rat apoE.

II.2.3.1.2. Agarose gel electrophoresis

Agarose gel electrophoresis was performed by Elliot Lee, a summer student to Dr. Yao. Conditioned serum-free media from cells expressing human apoB53, apoB72, and apoB100 were concentrated 10-fold using Centricon-10 concentrators (Amicon Inc., Beverly, MA). Volumes of 2 - 10 µl

were applied to agarose gels (Paragon, Beckman, Ltd.) and the lipoproteins resolved by electrophoresis for 60 minutes at 90 V according to the manufacturers' instructions. Lipoproteins in the resultant gels were transferred to nitrocellulose membranes for 40 - 45 minutes under 1 kg pressure and blotted for human apoB, rat apoE, or rat apoAI.

II.2.3.2. Immunoblot analyses

Immunoblotting can be divided into two steps: transfer of proteins from the gel to a nitrocellulose membrane, and detection of the protein of interest with specific antibody. Protein transfer is most commonly accomplished by electrophoretic methods to facilitate the mobilization of the protein from the gel to the membrane. Wet (tank) blotting, in which the gel-membrane is submerged in transfer buffer for electrotransfer, proved most effective for apoB proteins.

II.2.3.2.1. Transfer conditions

Proteins were transferred from the SDS polyacrylamide gel to a nitrocellulose membrane using a Bio-Rad electroblot system containing 0.025 M Tris-base, 0.192 M glycine, 20 % methanol (v/v) (transfer buffer) precooled to- and maintained with a cooling coil at- ~4 $^{o}$C. A constant voltage of 100 - 125 V was maintained for 5 - 6 hours. Methanol (20 %, v/v) was included in the transfer buffer in order to prevent the gel from swelling and to increase the binding capacity of the nitrocellulose membrane (Hames 1990). The use of nitrocellulose was preferred because it is relatively inexpensive, blocking from nonspecific antibody binding is fast and simple,

and detection of successful transfer can be easily accomplished by staining with the nonpermanent/removable protein dye Ponceau S.

Wet blotting was also performed for transferring rat apoAI and apoE to nitrocellulose. The only variation from the procedure outlined above was the time of transfer. For apoAI and apoE less time was required to complete the transfer because of their small size (28 kD and 34 kD, respectively) relative to the apoB species (200 kD - 550 kD). In fact, extended transfer times may result in loss of apoAI owing to penetration through the membrane.

II.2.3.2.2. Blocking, washes, incubation with antibodies and detection

Once the proteins have been transferred from the polyacrylamide gel to the nitrocellulose membrane, detection of specific proteins proceeds. This process is made up of four steps. In the first step the membrane is blocked. Blocking entails binding unoccupied regions of the membrane with nonreactive proteins in order to prevent nonspecific binding of antibodies to the membrane. In the second step the membrane is incubated with the first or primary antibody which recognizes the protein of interest. In the third step the membrane is incubated with a second antibody that recognizes the constant region of the first antibody. The second antibody is coupled to the enzyme horseradish peroxidase which, on reaction with a substrate, produces a product that can be visibly detected or can activate X-ray film. Thus the fourth step involves addition of the substrate and detection.

Enhanced chemiluminesence (ECL) was the system chosen because of its ease of handling, rapidity, sensitivity (as little as 1 pg of protein can be

detected), and avoidance of the hazards of working with radioisotopes. The technique also provides a "hard copy" of data, *i. e.*, by activating X-ray film which is then developed and fixed, a permanent record is produced. Blocking, washes and ECL detection were performed according to the manufacturers' instructions. Basically, a blot was treated with a blocking solution of 5 % dry milk powder in 2 mM Tris-base (pH 7.6), 137 mM NaCl (Tris buffered saline or TBS) for one hour at room temperature with shaking. The blot was then placed in a heat sealable bag with the appropriate primary antibody. Table II.1 lists the primary antibodies used in these studies. A primary antibody would be diluted 1:3000 in blocking solution and used to probe the blot 16 - 48 hours at 4 $^{o}$C. The blot would then be washed with TBS supplemented with 0.1 % (v/v) Tween 20 (poly(oxyethylene)$_{20}$-sorbitan-monolaurate) and incubated for 2 hours with the appropriate secondary antibody diluted 1:5000. In this study, horseradish peroxidase-conjugated goat anti-mouse IgG, which recognizes mouse monoclonal antibodies like 1D1 (Table II.1), or donkey anti-rabbit IgG, which recognizes antibodies from rabbit sera like 4496 (Table II.1), were used. The excess secondary antibody would then be washed off as for the first antibody and ECL detection performed.

II.2.3.3. Semiquantitation of cell and media lipoproteins

Immunoblotting was used to assess the steady state levels of apolipoproteins from different samples. Cells were plated onto 60 mm Primaria dishes (Becton-Dickson) in 3 ml G418 supplemented medium and incubated 16 - 24 hours at 37 $^{o}$C in 7 % $CO_2$. The medium was removed

and replaced with 3 ml serum-free DMEM and further incubated 16 - 24 hours (final cell confluency 80 %). Previously, it had been established that McA-RH7777 cells and stable lines could survive in the absence of serum for this length of time without ill effect (Blackhart *et al.* 1990, Becker *et al.* 1974). The conditioned medium was collected, mixed with 200 ml Cab-O-Sil suspension, and apolipoproteins recovered in 200 ml protein sample buffer (section II.2.3.1.1.). The cell layer was treated with 1 ml of the same protein sample buffer and scraped with a plastic scraper (Falcon) to obtain intact intracellular protein. The amount of medium and cell lysate loaded onto SDS polyacrylamide gels was based on cell protein as determined by the Lowry assay (section II.2.5.). The proteins were resolved by SDS PAGE (section II.2.3.1.1.) and subjected to immunoblotting (section II.2.3.2.) as described. In some instances the protein samples in the sample buffer were stored at -70 $^{o}$C and reheated prior to use. A Camag densitometer was employed to scan the bands visualized by the ECL detection system.

II.2.3.4. Lipoprotein isolation

II.2.3.4.1. Discontinuous density gradient ultracentrifugation

Lipoproteins were fractionated by ultracentrifugation a nonlinear salt gradient ranging from d=1.23 g/ml to 1.03 g/ml as described (Vance *et. al.* 1984, Yao and Vance 1988). Cell lines were plated onto 100 mm dishes (Falcon) and incubated with 8 ml serum-free DMEM for 16 hours once 60 - 70 % confluent. The media from two dishes were combined and centrifuged at 2500 rpm to remove cell debris. Protease inhibitors

phenylmethanesulfonyl fluoride (PMSF; 1 ml 15 % PMSF in dimethyl sulfoxide, w/v, per ml medium) and ethylenediaminetetraacetate (EDTA; 1 ml 0.5 M stock solution per ml medium) were then added to the cell-free media. Sodium chloride (3.76 g) was dissolved in the medium and this solution was carefully layered onto 1.6 g of sucrose which had been placed at the bottom of a 40-ml capacity quick-seal polyallomer tube (Beckman Instruments). Next, 3 ml of d=1.02 g/ml sodium bromide solution was layered onto the medium. The tube was then filled with a solution of 0.67 M sodium chloride, 0.05 % EDTA (w/v) and sealed. The tube was centrifuged in a Ti.70 rotor at 50 000 rpm for 42 hours at 10 $^{o}$C. Upon completion of the spin, the tube was carefully removed from the rotor and 20 2-ml fractions collected from a puncture at the bottom of the tube. The lipoproteins were concentrated, resolved, and subjected to immunoblotting as described above (section II.2.3.1.1. and II.2.3.2.).

II.2.3.4.2. Flotation ultracentrifugation

Separation of endogenous rat apoB100 and human apoB53 containing lipoproteins was accomplished by flotation ultracentrifugation. Four 60 mm dishes of cells (60 - 70 % confluent) were incubated in 3 ml serum free DMEM (per dish) for 24 hours and the media combined with solid potassium bromide to achieve a density of 1.05 g/ml. The adjusted density was verified gravimetrically. The media were loaded into 13 ml polyallomer tubes (Beckman Instruments), the tubes sealed, and centrifuged in a Ti. 70.1 rotor for 24 hours at 50 000 rpm (10 $^{o}$C). Two fractions were collected from a puncture at the bottom of the tube and the fractions were concentrated for

electrophoresis and immunoblotting as described above (section II.2.3.1.1. and II.2.3.2.). In some experiments the lipids were extracted from each fraction and subjected to high performance thin layer chromatography (HPTLC; see section II.2.3.5.2. and II.2.3.5.4.).

II.2.3.5. Lipid analysis

II.2.3.5.1. Metabolic labeling with [$^3$H]glycerol

[1(3)-$^3$H] glycerol has been used as a precursor for measurement of glycerolipid synthesis in rat hepatocytes (Davis 1986, Vance 1991). Therefore, cells (~80 % confluent, plated on Primaria 60 mm dishes) were washed twice with serum-free DMEM and given 1 ml DMEM supplemented with 10 μCi [$^3$H]glycerol. At 0, 1/2, 1, 2 and 3 hour time points, cells and media were collected for lipid extraction (section II.2.3.5.2.), resolved and quantitated by thin layer chromatography (TLC; section II.2.3.5.3.).

II.2.3.5.2. Lipid extraction

Lipids were extracted, either from media directly or else after adsorption of media lipoproteins with Cab-O-Sil, by chloroform/methanol (1:1, v/v). Cell lipids were obtained by collecting the cells in 2 ml ice cold methanol/water (1:1, v/v) to which 2 ml chloroform was added (final ratio of chloroform/methanol/water, 2:1:1, v/v/v). The lipids in the chloroform phase were washed twice with a "theoretical upper phase" (methanol/$H_2O$/chloroform, 48:47:3, v/v/v). Lipid extracts were dried under

nitrogen gas and either subject to thin-layer chromatography (section II.2.3.5.3.) or lipid mass analyses (section II.2.3.5.5.).

II.2.3.5.3. Thin layer chromatography

Lipids were resuspended in chloroform and spotted onto 20 X 20 cm silica plates. The plates were developed halfway in chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, v/v/v/v/v) to separate phospholipids, dried, then developed in hexane/diethyl ether/acetic acid (70:30:1, v/v/v) to the top of the plate to separate neutral lipids (Yao and Vance 1988). Lipid bands were visualized by iodine vapor. Some plates were initially scanned using a Bioscan-Hewlett Packard scanner or a whole lane was scraped and radioactivity measured in order to determine the distribution of radioactive precursor amongst the various lipid classes. The majority of the radioactivity was found in the positions corresponding to phosphatidylcholine and triglyceride standards (data not shown). Therefore, triglyceride and phosphatidylcholine bands were scraped from the plates into vials to which 0.5 ml water and 4.5 ml scintillation fluor (Ecolite, ICN Biomedicals, Inc.) were added. Radioactivity was quantified in a Beckman Liquid Scintillation counter after 2 - 4 days.

II.2.3.5.4. High performance thin layer chromatography

In some experiments the medium and cell lipids were spotted 1 cm from the base of 10 X 20 cm HPTLC plates that had been prerun in chlorofom:methanol:water (65:35:8, v/v/v) to remove contaminants that would interfere with the detection of cholesteryl ester at the solvent front.

The lipids were resolved in the solvents as for TLC (section II.2.3.5.3.) except that the second solvent system was made more apolar by increasing the proportion of hexane and reducing the diethyl ether correspondingly (final condition: hexane/diethyl ether/acetic acid, 80:20:1, v/v/v) to better resolve triglyceride and cholesteryl ester. Lipids were visualized by dipping the plate in a 3 % cupric acetate (w/v), 8 % phosphoric acid (v/v) solution and charring the plate at 180 $^{o}$C.

II.2.3.5.5. Lipid mass analysis

Lipid extracts were resuspended in 100 µl isopropanol, and 20 or 30 µl aliquots were tested for triglyceride, total cholesterol or phosphatidylcholine by enzymatic colorimetric assay. The assays were performed on microtitre plates and the standards (Wako Pure Chemical Industry, Ltd.) prepared in isopropanol. Plates were covered in parafilm and incubated at 37 $^{o}$C for the times specified by the manufacturer. Absorbances ($\lambda$=495 nm) were read with a plate reader. Initially, a quality control standard (human plasma provided by H. Czarnecka) was included to validate the assays.

II.2.3.6. Metabolic labeling with [$^{35}$S]methionine

The experiments described below were conducted in an enclosed chamber infused with 7 % $CO_2$ and maintained at 37$^{o}$C. Biopsy bags of charcoal were included to adsorb volatile $^{35}$S.

II.2.3.6.1. Synthetic rate experiments

The apoB synthetic rates were determined for a cell line expressing apoB53 by measuring incorporation of [$^{35}$S]methionine. Cells were plated on 60 mm dishes and incubated until 70 - 80 % confluent. The cells were washed twice with methionine-deficient DMEM and then incubated with 1 ml of the media supplemented with 200 µCi [$^{35}$S]methionine. Medium was aspirated at 2.5, 5, 10, 20 and 30 minutes and cellular apoB recovered by immunoprecipitation and processed as described below (section II.2.3.6.2.).

II.2.3.6.2. Pulse chase experiments

Pulse chase experiments were performed according to McLeod *et al.* (1994). Cells were plated on 60 mm dishes in 5 ml maintenance medium and incubated overnight as before (final confluency: 70 - 80 % on the day of the experiment). The dishes were then washed twice with serum-free, methionine deficient medium followed by the addition of 1 ml of the same medium supplemented with 200 µCi of [$^{35}$S]methionine for 2 hours. The cells were then chased for up to 4 hours with DMEM. At the indicated time points cellular (diluted 1:5) and medium human and rat apoB was quantitatively immunoprecipitated with polyclonal sheep anti-human apoB serum (Boehringer Mannheim). The sheep antibody recognizes both human and rat apoB proteins. Protein A-Sepharose CL-4B was used to concentrate the immunocomplexes. The beads were washed six times with Radioimmune Precipitation (RIPA) buffer consisting of 0.001 M EDTA, 0.05 M Tris pH 8, 1 % deoxycholate (w/v), 0.1 % polyoxyethylene [9-10] p-t-octyl

phenol (Triton X-100) (v/v), 0.001 M DTT, 0.15 M NaCl, 0.015 % PMSF (w/v) and supplemented with 0.1 % SDS (w/v). ApoB was eluted from the beads with 8 M urea, 2 % SDS (w/v), 10 % glycerol (v/v), 5 % b-mercaptoethanol (v/v), 0.005 % BPB (w/v) (RIPA sample buffer) and subjected to SDS PAGE as described (section II.2.3.1.1.). The gels were stained, destained, and treated with the fluorographic reagent Amplify (Amersham), dried, and exposed to X-ray film at -70$^{o}$C for 16 hours. The bands corresponding to the cell and medium apoB proteins were then cut out, digested with 0.5 ml hydrogen peroxide at 75 $^{o}$C for 16 hours and counted in 10 ml Hionic-Fluor (Canberra) quenched with 0.25 ml perchloric acid.

For apoB53 experiments, pulse chase was performed as above with the modification that the length of the pulse was 30 minutes followed by chase medium consisting of 2 mM "cold" methionine in DMEM.

II.2.3.6.3. Reuptake experiments

Reuptake experiments were performed on a cell line expressing apoB53 using chase medium from a 100 mm dish of 70 - 80 % confluent cells pulsed with 1.1 mCi of [$^{35}$S]methionine (per 5 ml methionine deficient media) for 2 hours followed by a 2 hour chase of 2 mM unlabeled methionine supplemented DMEM. Cells and media were collected at the indicated time points, immunoprecipitated and processed as described for the pulse chase experiments.

II.2.4. Isolation of RNA and RNase protection

RNase protection assay was performed by Alan Brookes at the Gladstone Institute. Briefly, total RNA was prepared from the cell lines by the method of Chirgwin *et al.* (1979). Fifteen micrograms of total RNA was hybridized to a $^{32}P$ uniformly labeled antisense RNA probe encompassing sequences from -85 to +121 of the human apoB gene in a buffer containing 80% formamide, 40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA at 55 $^{o}C$ for 16 hours. The hybrids were then digested with ribonuclease T1 at a final concentration of 4 μg/ml for 1 hour at 30 $^{o}C$. The reaction was terminated by addition of SDS and proteinase K and incubated at 37 $^{o}C$ for 15 minutes. After extraction with phenol/chloroform the RNA was recovered by ethanol precipitation using 10 mg of yeast transfer RNA as carrier, dissolved in formamide loading buffer, and fractionated on an 8 % polyacrylamide sequencing gel.

II.2.5. Cell protein isolation and protein assay

For most experiments, one dish of cells was reserved for determining protein concentration. Cells were washed twice with PBS followed by incubation in 1 ml 0.1 N NaOH for 60 mm dishes or 4 ml 0.1 N NaOH for 100 mm dishes. The dishes were then agitated at room temperature for 30 minutes to hydrolyze the cell proteins. The lysate was collected and the dishes rinsed with another 1 ml 0.1 N NaOH which was pooled with the first (the rinse step was not performed on 100 mm dishes). The samples were thoroughly mixed before aliquots of 50 μl were assayed for protein

concentration by the method of Lowry *et al.* (1951) using BSA standards of 6.25, 12.5, 25, 50 and 75 µg. Absorbance readings at 660 nm and standard curve were obtained using a Hitachi spectrophotometer. On some occasions the lysates were stored at 4 $^{o}$C for 1 - 2 days before the assay was performed.

**Figure II.1:** Structure of expression plasmids encoding human apoB53, apoB64, apoB72 and apoB100 (Yao *et al.* 1992, McLeod *et al.* 1994). Thick lines designate apoB100 cDNA cut with restriction enzymes to generate the coding regions of the nucleotide (nt) length specifed. Codon 2153 (white square) was changed from CAA (Gln) to CTA (Leu) to preclude the formation of apoB48 (see text). Thin lines denote pCMV5, the expression vector used. AMP, ampicillin resistance gene, CMV, cytomegalovirus promoter and enhancer sequences, hGH human growth hormone transcription termination and polyadenylation signals, ATG, translational start site of apoB100.

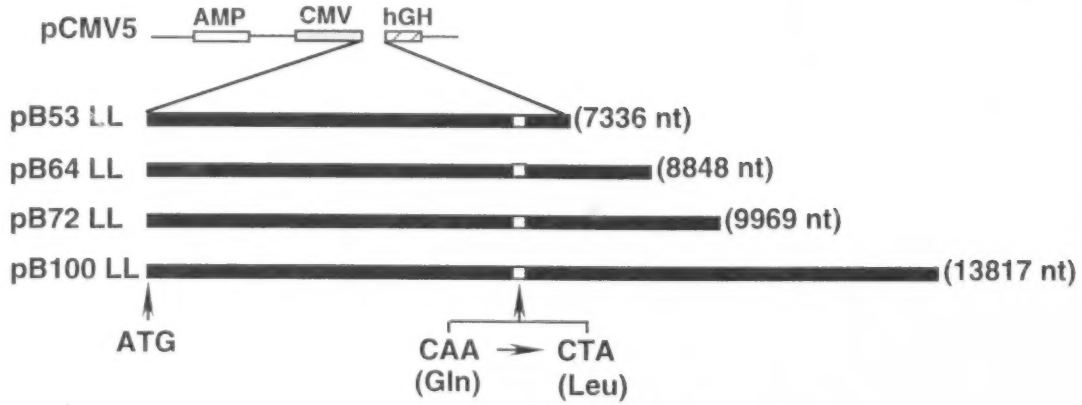


**Figure II.2:** Length of the proteins encoded by pB53 LL, pB64 LL, pB72 LL and pB100 LL (Yao *et al.* 1992, McLeod *et al.* 1994). Scale indicates percentage of apoB100 the protein represents. The number of amino acids (a. a.) predicted to be found in each protein is given in parentheses. The square brackets encompass the epitopes recognized by the monoclonal antibodies 1D1, 3F5, and 4G3.

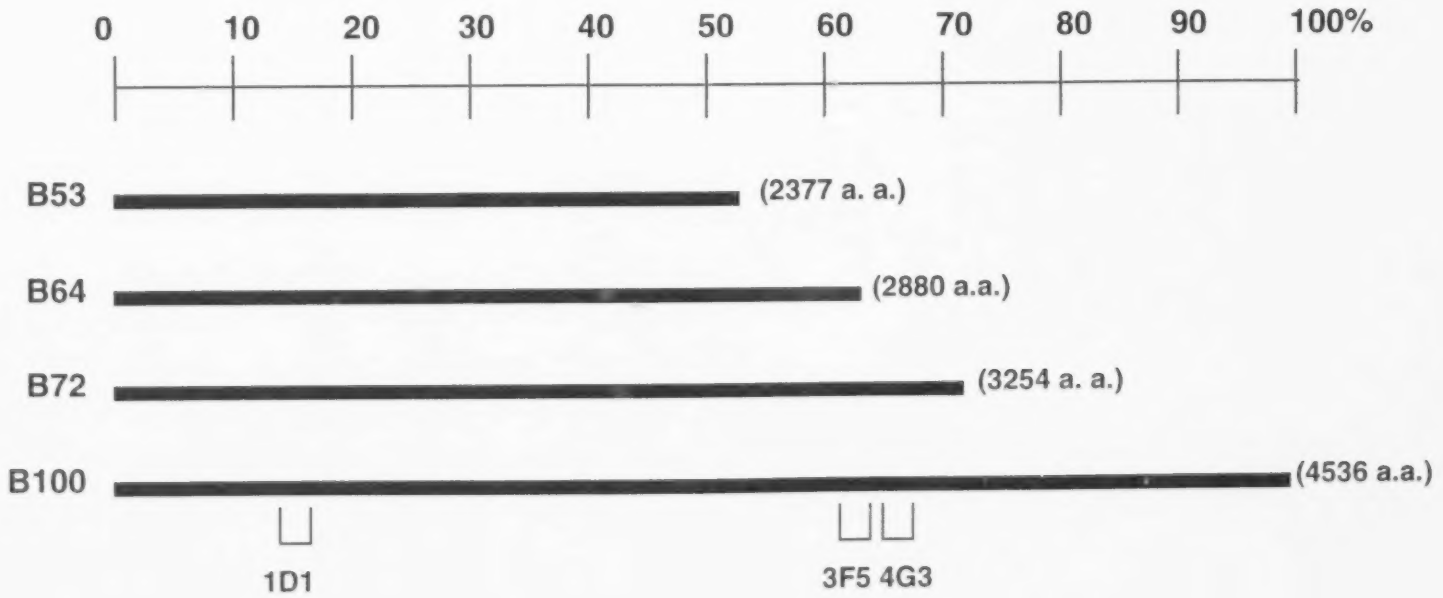


**Table II.1:** Primary antibodies and antisera used for Western blot analyses

| antibody/ antiserum | source | derivation | comments |
|---|---|---|---|
| 1D1 | mouse monoclonal | human LDL | recognizes a.a. 474 - 539 of human apoB100* |
| 3F5 | mouse monoclonal | human LDL | recognizes a.a. 2835 - 2922 of human apoB100* |
| 4G3 | mouse monoclonal | human LDL | recognizes a.a. 2980 - 3084 of human apoB100* |
| 2140 | rabbit polyclonal | human apoB100 peptide (a.a. 2140 - 2151) | recognizes human apoB48; does not cross react with human apoB100** |
| 4496 | rabbit polyclonal | rat LDL | recognizes rat apoB100 and apoB48; does not cross react with human apoB species*** |
| anti-AI | rabbit polyclonal | human apoAI | cross reacts with rat apoAI**** |
| anti-E | rabbit polyclonal | human apoE | cross reacts with rat apoE**** |

*Pease *et al.* 1990
**Blackhart *et al.* 1990, Yao *et al.* 1992
***R. Davis, personal communication; rabbit antiserum was subjected to an affinity column of human LDL, antibodies recognizing human LDL were retained so the eluant was essentially free of cross reactive antibodies
****T. Ohnishi, personal communication

# Chapter III. EFFECT OF CARBOXYL-TERMINAL TRUNCATION ON APOB-CONTAINING LIPOPROTEIN ASSEMBLY AND SECRETION*

## III.1. INTRODUCTION

Familial hypobetalipoproteinemia (hypobeta) is a rare codominant autosomal disorder associated with mutations of the apoB gene. Homozygous individuals have very low or undetectable levels of apoB100 proteins in the plasma and may have profound malabsorption of fat and fat-soluble vitamins. In the heterozygous state the affected persons have somewhat reduced levels of plasma apoB compared to normal (plasma apoB100 concentrations ranging from less than one quarter to one half that of normal). Over 20 truncation variants of apoB100 have been found; they are caused by framsehift or point mutations that result in the formation of stop codons within the apoB transcript.

The exact mechanism by which truncated variants of apoB100 are linked to the hypobeta phenotype remains unclear. It has been observed that the truncated forms of apoB100 are associated with small, dense particles (*i. e.*, demonstrating high protein to lipid ratios) in the plasma. Metabolic labeling studies performed on several heterozygous hypobetalipoproteinemic patients indicate that the rate of secretion of the truncated apoB may be reduced, perhaps suggesting that the truncated apoB species may not be adequate for the assembly of normal triglyceride

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rich lipoproteins (Krul *et al.* 1992). Alternately, some truncated forms of apoB100 could be rapidly cleared from the plasma (reviewed Linton *et al.* 1993a).

To determine the regions of apoB100 necessary for lipoprotein assembly and secretion, a number of investigators developed a series of recombinant carboxyl-terminal truncated apoB expressing cell lines (Yao *et al.* 1991, Graham *et al.* 1991, Spring *et al.* 1992a and b). Yao *et al.* (1991) observed that the length of apoB inversely correlated with the density of the particle it was associated with, *i. e.*, the shorter the apoB variant, the denser the lipoprotein particle. However, although an inverse relationship between apoB length was observed ranging from apoB18 to apoB53 (representing the amino-terminal 18 to 53 % of the full length protein) and the lipoprotein buoyant density, it is unknown if this relationship holds for larger apoB species. Furthermore, hepatoma cell lines transfected with shorter apoB forms such as apoB37 demonstrated efficient secretion of the truncated apoB protein, suggesting that low plasma levels observed in hypobeta may not be a consequence of diminished secretion. It is unknown if such is the case for the longer apoB variants.

In this study, stably transfected McArdle-RH7777 cells that synthesize and secrete carboxyl-terminal truncated human apoB64, apoB72 or full length apoB100 were generated. The effect of truncation on the assembly and secretion of hepatic lipoproteins was analyzed. It was found that the apoB-containing lipoproteins exhibit densities that decrease with increasing apoB length, as observed in hypobeta. Furthermore, the kinetics of

secretion of the apoB proteins were nearly as efficient as that of the endogenous apoB100. Therefore, low levels of plasma apoB observed in hypobeta may not be due to impaired secretion.

## III.2. RESULTS

### III.2.1. Generation of transfected McA-RH7777 cells expressing recombinant human apoB64, apoB72, and apoB100

McA-RH7777 cells were transfected with the plasmids encoding human apoB64, apoB72 or apoB100 as described in chapter II. Part of the screen for positive clones expressing apoB64 is shown in Figure III.1. Out of thirty G418 resistant clones tested, 2 clones (B64-6 and B64-13) were found to secrete an immunoreactive protein of the expected size of apoB64 (*i. e.*, ~317 kD), although additional immunoreactive degradation products were also observed. Notably, despite the attempt to eliminate formation of apoB48, a protein approximately the same size as apoB48 (~220 kD, designated B48* in Figure III.1) was detected.

Three clones secreting different levels of human apoB72 (B72-21, B72-33, and B72-38) and apoB100 (B100-18, B100-24, and B100-36) were also obtained (Figure III.2 A.). Degradation products and B48* found in B64-6 and B64-13 were also observed in these clones.

The steady state levels of secreted rat apoB did not appear to be affected by the transfection (data not shown) or by the level of expression of human apoB72 and apoB100 as determined by immunoblot analysis

(Figure III.2 B.). For example, densitometric analysis of the steady state levels of the rat apoB100 in clones expressing human apoB100 revealed that the steady state levels were similar to those of nontransfected cells (*i. e.*, demonstrating a ratio of 1.1:1.1:1.3:1, B100-18:B100-24:B100-36:McA-RH7777; data not shown). A clone expressing human apoB64 (B64-17, provided by Yang Zhao) was also examined. The levels of endogenous apoB in apoB64-transfected cells were similar to the apoB72 expressing clones (data not shown).

III.2.2. Verification of the recombinant proteins expressed by stably transfected McA-RH7777 cells

The carboxyl terminus of each mutant apoB species was verified by epitope mapping to ensure that the correct apoB reading frame had been maintained in all the expression plasmids. For example, apoB64 was tested with monoclonal antibodies 3F5 and 4G3 that recognize epitopes around residues 2900 of full length apoB100 (Pease *et al.* 1990). The antibodies appeared to detect apoB72, as expected, but did not detect apoB64 or other short species such as apoB60 or apoB53 (Figure III.3 panel 3F5 and 4G3). ApoB48* was tested for identity to apoB48 using an antibody specific to the carboxyl terminus of authentic human apoB48 that does not cross react with rat apoB48 or human or rat apoB100. Immunoblot analysis (Figure III.3 panel 2140) clearly demonstrated that the apoB48* protein secreted by the pB64 LL transfected cells did not react with this antibody. ApoB72 and apoB100 produced by the corresponding cell lines were similarly authenticated (McLeod *et al.* 1994).

The apoB48* proteins expressed by all of these lines appear to be derived from the amino-terminal portion of apoB100 since the protein reacted with antibodies specific for more amino-terminal epitopes such as 1D1 but not with antibodies specific for more carboxyl-terminal epitopes of apoB100 like 3F5. The mechanism of the apoB48* protein production in McA-RH7777 cells has been investigated (Johnson and Innerarity 1993, Heinemann *et al.* 1994); it is thought to be due to utilization of a cryptic polyadenylation signal (nucleotide position 6752 of the human apoB100 cDNA) such that addition of a polyA tail 10 nucleotides downstream of the signal results in the formation of a stop codon (UAA) in the transcript.

III.2.3. Characterization of lipoproteins containing the human apoB variants

The buoyant density of lipoproteins containing the recombinant apoB64, apoB72 or apoB100 was determined by density gradient ultracentrifugation (Figure III.4 A.). Each apoB species exhibited a characteristic density distribution. For example, apoB64 distributed over a wide density range with a major peak at ~1.06 g/ml, whereas apoB72 and apoB100 were found within narrow ranges that peak at d=1.04 and d=1.03 g/ml, respectively. In all stable cell lines tested, the apoB48* protein was found predominantly in the HDL density range.

The position of endogenous rat apoB was determined by probing the blots shown in Figure III.4 A. with antibody specific for rat LDL (see chapter II, Table II.1, antiserum 4496). The density distribution of rat apoB100 or apoB48 was not altered by the production of the recombinant apoB species

(Figure III.4 B., compare recombinant apoB cell lines to untransfected cell line). Rat apoB100 and apoB48 were located mainly in the d=1.03 and 1.10-1.19 g/ml density range, respectively, as observed previously (Tanabe *et al.* 1989, Yao *et al.* 1993). The density distribution of apoB72 and apoB100 was also examined in the multiple cell lines that secreted different levels of the same apoB form (*e. g.* clones B72-38 and B72-33, shown in Figure III.2). It was found that the level of secretion had no effect on the buoyant density (B72-38 and B100-24 are shown in Figure II.4, B72-33 and B100-18 are reported in McLeod *et al.* 1994; see also chapter IV). Together the results suggest that recombinant human and endogenous rat apoB proteins are discrete lipoprotein particles and that particle density is determined by intrinsic properties of apoB proteins.

The lipoproteins produced by the cells producing human apoB72 or apoB100 were further characterized by agarose gel electrophoresis and immunoblot analyses (Figure III.5). It was found that the apoB-containing lipoproteins exhibited b mobility, similar to human LDL (Figure III.5, A.) and endogenous rat apoB-containing lipoproteins (Yao *et al.* 1993), and they contained little or no apoAI or apoE (Figure III.5 B. and C.). This result suggests that the apoB-containing particles produced by these cells are distinct from those containing apoAI or apoE.

Previous work with shorter apoB truncation mutants revealed an inverse relationship between the length of apoB and lipoprotein density (Yao *et al.* 1991). When the peak density values of apoB64, B72 and B100 were plotted together with the values for the shorter apoB truncation mutants

against the percentage length of apoB, an inverse linear relationship was observed extending from apoB23 to apoB53, and from apoB64 to apoB90 (Figure III.6). However, between apoB53 and apoB64, lipoprotein density appears to be independent of the length of apoB, suggesting that this region may not be directly involved in the initial stages of lipoprotein assembly.

Overall, these results support the notion that the length of apoB has an important effect on the structure of apoB-containing lipoproteins and that the recombinant human and endogenous rat apoB species form discrete lipoproteins.

III.2.4. Kinetics of synthesis and secretion of apoB species produced by the stably transformed cells

The kinetics of apoB synthesis and secretion by the stably transfected cell lines were analyzed by pulse chase experiments. These experiments permitted determination of the intracellular retention half-time ($t_{1/2}$, defined as the time at which 50 % of the maximally labeled apoB is present within the cell) and secretion efficiency of apoB (defined as the % of the maximally labeled apoB secreted by the end of the chase period). Nontransfected McA-RH7777 cells had been utilized to establish the kinetics of rat apoB100 (Yao *et al.* 1991, McLeod *et al.* 1994). A characteristic feature of apoB100 secretion is that the decrease in cellular radiolabel with time is not accompanied by a concomitant increase in the amount of radiolabeled apoB100 secreted, *i. e.*, while ~20 % of the initial apoB100 label was found in the medium after 4 hours, ~20 % remained within the cells. The

remaining ~60 % of apoB100 had been degraded without secretion. Similar kinetics have been observed in HepG2 cells (for example see Boström *et al.* 1988) and primary rat hepatocytes (Borchardt and Davis 1987).

Pulse chase experiments of a clone expressing human apoB72 are shown in Figure III.7. Notably, the $t_{1/2}$ for human apoB72 in clone B72-38 (Figure III.7 A. top panel, circles) was ~1.5 hours while the $t_{1/2}$ for endogenous rat apoB100 of this cell line (Figure III.7 B. top panel, circles) was ~1.0 hours, similar to that of apoB100 in nontransfected McA-RH7777 cells (Yao *et al.* 1991, McLeod *et al.* 1994). The secretion efficiency of recombinant apoB72 was similar to that of endogenous apoB100 of nontransfected McA-RH7777 cells: only ~20 % of the total apoB label was recovered in the medium by the end of 4 hours (Figure III.7 A. and B. middle panels, circles). Furthermore, ~50 % of the total label (cell plus medium) in apoB72 was recovered at this time (Figure III.7 A. bottom panel), similar to the endogenous rat apoB100 of this cell line (Figure II.7 B. bottom panel, black bars). Thus ~50 % of the radiolabeled apoB72 was unaccounted for and represents the portion of apoB72 that was degraded intracellularly. The other human apoB72 expressing cell lines were also studied. ApoB72 and endogenous rat apoB100 of clone B72-33 exhibited kinetics similar to those of clone B72-38 (McLeod *et al.* 1994). While the level of apoB72 produced by clone B72-21 was too low to be detected by pulse chase analysis, the clone could be used to assess endogenous rat apoB100 kinetics. As shown in Figure III.7 B. (top and middle panel, squares), endogenous rat apoB100 in clone B72-21 may have a slightly longer intracellular retention time ($t_{1/2}$

of ~1.25 hours) and have a slightly reduced secretion efficiency (19 % *versus* 23 %, B72-21 and B72-38, respectively) compared to rat apoB100 in clone B72-38 (Figure III.7 B. top and middle panel, circles) although the total amount of apoB100 recovered was similar between the two clones (Figure III.7 B. bottom panel). Therefore, it appeared that absence of the carboxyl-terminal 28 % apoB100 did not impair secretion of apoB mutants inasmuch as the kinetic pattern of apoB72 resembled endogenous rat apoB100. Finally, the synthesis and secretion of human apoB72 did not affect the pattern of apoAI secretion in these cells since the apoAI kinectics were similar to that observed in nontransfected McA-RH7777 cells (McLeod *et al.* 1994), *i. e.*, demonstrating a $t_{1/2}$ of ~1.0 and ~1.25 hours in B72-38 and B72-21, respectively, and a secretion efficiency of ~35 % in both cell lines (Figure III.7 C.).

Pulse chase experiments were also performed on two of the stable transformants expressing human apoB100, cell lines B100-18 and B100-24. Since the polyclonal antibodies used in the pulse chase experiment quantitatively immunoprecipitate both human and rat apoB species, the human recombinant apoB100 could not be resolved from the rat endogenous apoB100, therefore the kinetic pattern representing both species simultaneously was determined (Figure III.8). Notably, the clone expressing higher steady state levels of human apoB100 (B100-18 in Figure III.2; in Figure III.8, circles in top and middle panels, dark bars in bottom panel) demonstrated a relatively shorter intracellular retention time ($t_{1/2}$ of ~1.25 hours) compared to the low expressor (B100-24 in Figure III.2; in

Figure III.8, squares in top and middle panels, and light bars in bottom panel; $t_{1/2}$ of ~1.5 hours) yet secreted more apoB protein into the media over at least the initial 2 hours of the chase period. Presumably the clone secreted more human apoB100 into the media since steady state levels of intracellular and secreted endogenous apoB100 were similar for both the high and low expressing human apoB100 clones (Figure III.2 B.). The effect of expression of human apoB100 on the synthesis and secretion of endogenous rat apoB100 merits further investigation once the tools are available to distinguish the human from the rat apoB100 proteins.

Pulse chase analysis of a human apoB64 expressing cell line suggested that human apoB64 was retained longer within the cell ($t_{1/2}$ of ~2.0 hours) and was secreted as efficiently as apoB100 produced by untransfected cells but without as much loss of signal due to degradation (Figure III.9 A.). In this regard human apoB64 may resemble rat apoB48 kinetically (Borchardt and Davis 1987, Sparks and Sparks 1990). Endogenous apoB100 produced by this clone demonstrated a half-life of ~1.5 hours and a secretion efficiency of 40 % by the end of the chase period (Figure III.9 B.).

In sum, the results of pulse chase analyses suggest that human apoB species can be secreted as efficiently as rat apoB proteins.

## III.3. DISCUSSION

### III.3.1. Summary

Rat hepatoma cell lines stably expressing human apoB64, apoB72 or the full length apoB100 were generated in order to study the effects of carboxyl-terminal truncation on the structure and secretion of apoB-containing lipoproteins and to determine if their behavior *in vitro* resembles the *in vivo* situation depicted by the disease hypobetalipoproteinemia. It was found that the inverse relationship between the length of apoB and the buoyant density of the lipoprotein particle may apply from apoB23 to apoB53 and from apoB64 to apoB90. Furthermore, the secretion of human apoB from the transformed cells is not impaired by carboxyl-terminal truncation: apoB64 and B72 (and presumably human apoB100) are secreted as efficiently as rat apoB100.

### III.3.2. Length of apoB determines lipoprotein density

ApoB100 is a large hydrophobic protein shown to be necessary for VLDL assembly. The amino acid sequence of apoB100 consists of numerous hydrophobic regions distributed throughout its length. These regions are thought to serve in the assembly of apoB100-containing lipoproteins by binding lipid. The importance of these regions has been borne out by studies on carboxyl truncated species of apoB100: truncation decreases the number of lipid binding regions available for lipid recruitment thus resulting in more dense, lipid-poor lipoproteins. However, while the

length of apoB seemed to dictate the amount of lipid associated with the newly assembled lipoprotein up to and including the entire length of the protein, the results of the current study suggest that only specific regions may be of importance with respect to lipoprotein assembly, *i. e.*, the regions between apoB23 and apoB53 and between apoB64 and apoB90. The reason for this unexpected finding is not entirely known. Recently, Segrest *et al.* (1994) have determined that apoB100 has a pentapartite structure composed of three amphipathic $\alpha$ helical regions that alternate with two amphipathic $\beta$ strand regions: $NH_2$-$\alpha_1$-$\beta_1$-$\alpha_2$-$\beta_2$-$\alpha_3$-COOH; $a_1$ represents the globular amino-terminal residues 58 through 476; $\alpha_2$, 2103 through 2560; and $\alpha_3$, 4061 through 4338 of apoB100 (shown schematically above plot in Figure III.6). The 5 part division of apoB100 based on this computer analysis is in good agreement with that achieved by trypsin releasability studies (Yang *et al.* 1989) and other computer analyses (De Loof *et al.* 1987). Therefore, it is proposed that the density-length relationship observed previously may hold for regions predicted to have high $\beta$ strand potential such as between apoB23 and apoB53 (residues 1003 - 2377) and between apoB64 and apoB90 (residues 2880 - 4082), although further dissection of apoB may be required to be certain. It has been postulated that the regions with high amphipathic $\beta$ strand content may represent irreversible lipid-binding regions of apoB100 while the amphipathic $\alpha$ helical regions may represent reversible, flexible, lipid-binding regions (Segrest *et al.* 1994). The irreversible nature of the $\beta$ strand regions may therefore be what confers lipoprotein structure during the initial stages of assembly. The results of the current study also suggest that the density of apoB-containing

lipoproteins may be independent of apoB length within the region between apoB53 and apoB64 (residues 2377 - 2880), although again, further dissection of apoB may be required to be certain. This region may represent a transition point and corresponds to the second region predicted to have high $\alpha$ helical content ($\alpha_2$) and to just bridge the second $\beta$ strand region ($\beta_2$) based on the computer model. Therefore, although a uniform distribution of lipid binding regions has been demonstrated for apoB100, not all may be required for the initial stages of lipoprotein assembly.

Given the inverse relationship between apoB size and lipoprotein density shown in the transfected cells, then why have some of the truncated apoB species of hypobeta been found to be associated with more buoyant lipoproteins (Young 1990)? For example, LDL-like particles are secreted with amino-terminal fragments as small as apoB32 (McCormick, *et al.* 1992). Moreover, apoB48 is a necessary component of intestinally derived chylomicrons and is secreted as part of VLDL by rat hepatocytes. However, in nearly all instances that a truncated apoB species is detectable in more buoyant lipoproteins, the truncated apolipoprotein can also be detected in smaller, denser particles which demonstrate the density dependence on length of the truncated apoB (Linton *et al.* 1993a). In addition, a portion of apoB48 produced by rat hepatocytes or perfused rat livers is also secreted as HDL sized lipoproteins (Borén *et al.* 1994; references cited within). The density-length relationship may reflect a fundamental stage of apoB-containing lipoprotein assembly. Thus apoB is co-translationally associated with lipid, the degree of lipidation dictated by the amount of apoB translated

and translocated. Once translation of the apoB peptide chain is completed and the protein is fully completely translocated, then a second step occurs in which the nascent lipoprotein may fuse with a preformed lipid droplet to result in the formation of VLDL (Schumaker *et al.* 1994, Borén *et al.* 1994). HepG2 and perhaps McA-RH7777 cells may be deficient in the second step thus they secrete very few lipoproteins in the VLDL density range (Spring *et al.* 1992b). The mechanism underlying this defect is unknown. Since neither cell line appears to be deficient in neutral lipids, such as triglyceride (Dixon and Ginsberg 1993; Morton *et al.* 1977), one possibility may be an inability to mobilize fatty acids produced by lipolysis of cytosolic stores of triglyceride. Fatty acid mobilization from cytosolic stores may be required for synthesis of triglyceride destined for secretion with apoB-containing lipoproteins (Wiggins and Gibbons 1992). The activity of the enzymes involved in de-esterification (lipases) and/or re-esterification (fatty acid activating enzymes, acyl transferases) may be compromised in the hepatoma cell lines. HepG2 cells do appear to demonstrate limited lipolysis compared to rat hepatocytes (Gibbons *et al.* 1994). McA-RH7777 cells, in contrast, have been reported to have enhanced lipolytic activity but reduced ability to activate the fatty acids produced so that the re-esterification process may not occur as readily as for rat hepatocytes (Morton *et al.* 1977, Morton *et al.* 1982).

The density distribution of human apoB23-, apoB32-, apoB36- and apoB41-containing lipoproteins produced by stably transfected McA-RH7777 cell lines can be influenced by the presence of oleate (White *et al.*

1992). Although most of the truncation mutants in this study demonstrated greater lipidation (thus greater buoyancy) on supplementation with oleate, none were found in the VLDL range of the density gradient employed. This could be due to a constraint of the length-density relationship of lipoprotein assembly, at least for apoB truncation variants in the range tested, or a consequence of the culture conditions utilized. In other studies with oleate supplementation, it was found that the number of apoB-containing lipoproteins secreted by HepG2 was increased but the lipoprotein size was minimally affected (Sniderman and Cianflone 1993). Similar results are obtained in liver perfusion studies, provided the liver is obtained from a fasted rat (Dixon and Ginsberg 1993). If a liver is obtained from a fed rat, it secretes the same number of apoB-containing lipoproteins in the presence or absence of fatty acid but the lipoproteins are more buoyant, lipid rich particles consequent to fatty acid treatment (Gibbons 1990). HepG2 cells supplemented with glucose also secrete more buoyant lipoproteins (Sniderman and Cianflone 1993). Thus hepatoma cells may reflect mechanistic changes in lipoprotein assembly (*e. g.* second step shut down?) dependent on nutritional status - *i. e.*, they may represent cells "frozen" in a fasted metabolic state that divert the neutral lipid pools for utilization by the cell (membrane synthesis or energy production) rather than for secretion in a lipoprotein. Substrate availability may not take precedence, however, since it is shown in chapter IV that increased expression of apoB results in increased secretion.

The need for a second step in VLDL synthesis is not entirely overcome by expression of microsomal triglyceride transfer protein (MTP), a protein thought to be of great importance in the assembly of apoB-containing lipoproteins (Gregg and Wetterau 1994). Heterologous cells made to coexpress apoB41 (Leiper *et al.* 1994) or apoB53 (Gordon *et al.* 1994) with MTP do not form VLDL containing these apolipoproteins. Therefore, additional factors may be required. ApoB48, which is secreted primarily as an HDL sized particle by McA-RH7777 cells, can be secreted to a large extent as VLDL on provision of oleate (Borén *et al.* 1994). Could this indicate that the second step model may apply only to apoB48 lipoprotein assembly? ApoB48 displays differences in cellular and secretory kinetics (Borchardt and Davis 1987), responsivity to various stimuli such as insulin (Sparks and Sparks 1990), and its secretion appears not to be entirely dependent on *de novo* protein synthesis like apoB100 (Borén *et al.* 1994). Therefore, apoB48 may be synthesized and secreted by a mechanism that differs from that of apoB100 and as such may not be governed by the same "rules" such as the length-density relationship observed for apoB100. It would be of interest to examine the effects of fatty acid provision on the mutant variants of apoB other than apoB48 in order to further elucidate the mechanism of VLDL formation in McA-RH7777 cells.

Finally, the current studies with carboxyl truncation of apoB do not indicate how the apoB peptide becomes associated with lipid, *i. e.*, if this event is mediated by chaperones or by direct interaction of apoB with MTP.

Further studies employing these apoB truncation variants may provide insight into these mechanisms.

III.3.3. Carboxyl truncation of apoB does not impair secretion of lipoproteins

Low plasma levels of apoB are invariably observed in patients with hypobeta (Linton *et al.* 1993a). This observation could be attributed to the secretion of decreased numbers of apoB particles due to alterations at one or more sites of regulation, *e. g.*, changes in levels of apoB mRNA or protein synthesis, or due to co- or post-translational events such as translocation across the ER membrane or degradation within the ER. While the current work is by no means definitive for hypobeta, the observation that the secretion efficiency (*i. e.*, amount secreted *versus* degraded) of the carboxyl truncation mutants produced by the stable transformants was similar to endogenous rat apoB suggests that truncation does not specifically impede apoB protein secretion. Therefore the defect in apoB metabolism underlying hypobeta may not be due to a defect in apoB secretion.

In 1990, Ross *et al.* reported a reduction in the steady state levels of apoB mRNA in a homozygous hypobeta patient. A reduction in apoB mRNA levels has also been observed in mice in which the apoB allele was modified so that it coded for a truncated mouse apoB protein (Homanics *et al.* 1993). The mice demonstrated a reduction in plasma apoB levels, similar to hypobeta. In addition, however, the mice also displayed hydrocephaly and encephaly which had not been seen in human patients. While the gene modification may have imposed a position effect as suggested by the

investigators, these results may reflect a need for apoB in mouse development that may have been lost in humans. It has been observed that the steady state levels of most cellular or viral mRNAs that contain either a nonsense or frameshift mutation are dramatically reduced in most eukaryotes (Pulak and Anderson 1993; references cited within). It is currently unknown exactly how the premature stop codon confers destabilization of the transcript that contains it. Current work suggests that a nuclear surveillance system may be responsible for monitoring and degrading the "faulty" mature transcripts during export from the nucleus (Pulak and Anderson 1993; Belgrader *et al.* 1994). The half-life of full length apoB mRNA in HepG2 cells is 16 h (Pullinger *et al.* 1989). However, the decay kinetics of apoB mRNA have not been examined in hypobeta or in the hypobeta mouse model. The human apoB constructs used in this study were not designed to have premature stop codons within the apoB coding region to generate the truncated apoB proteins. It may be of interest to modify the human apoB constructs or even the rat apoB gene in McA-RH7777 cells to address the role of apoB mRNA stability in hypobeta.

Most evidence supports the role of the ER as rate-limiting in the synthesis and secretion, as well as is the site for degradation of apoB-containing lipoproteins (Borchardt and Davis 1987, Sato *et al.* 1990, Furukawa *et al.* 1992). It could well be that truncated species of apoB, particularly the shorter species, are recognized as malformed in the ER and are subsequently degraded rather than secreted. It is also possible that the human apoB species are not recognized by the proteases involved in

degradation of rat apoB. A defect or inability to recognize the human apoB seems unlikely though, since human apoB demonstrated kinetics similar to rat apoB (although it is possible that they involved separate mechanisms to achieve the same effect). Human apoB and rat apoB may be sufficiently similar to discount species differences (Li *et al.* 1988, Knott *et al.* 1985).

In hypobeta heterozygotes it is invariably observed that there are not only low levels of the truncated apoB species in the plasma but also low levels of apoB100 relative to normal unaffected individuals. Work done by Krul *et al.* (1992) and Aguilar-Salinas *et al.* (1995) suggests that this may in part be due to decreased levels of production of the full length protein. How one mutant apoB allele could exert this effect on the wild type allele is currently unknown. One possibility is that shorter forms of apoB message/ribosome complexes could outcompete the normal message/ribosome complexes for translocation into the ER. Reduction in rat apoB100 mass and synthesis was observed on expression of human apoB48 and apoB18 (Hussain *et al.* 1995). In this study, however, there did not appear to be an effect on the steady state levels or secretion efficiency of endogenous apoB consequent of the expression of human apoB64, apoB72 or full length apoB100. This result may reflect again, an apoB length-dependence for effect. The effects of carboxyl truncated apoB on full length apoB100 production merit further investigation.

**Figure III.1:** Screen for stable transformants of McA-RH7777 cells that secrete recombinant human apoB64. McA-RH7777 cells were cotransfected with pB64 LL and pSV2neo and 48 hours later the media was changed to media containing G418. Two-three weeks later G418 resistant colonies were transferred to 6 well dishes and grown to 60-70 percent confluency. The culture media from each colony was collected, the lipoproteins concentrated and resolved by electrophoresis on 5 % polyacrylamide 0.1 % SDS gels. Proteins were transferred to nitrocellulose membranes and probed with an antibody specific for human apoB (1D1; see Table II.1, chapter II.). Media from cells known to be expressing human apoB53 (lane pB53) was used for a size marker and positive control for the antibody. Positions of apoB53 along with the apoB64 species expected are indicated on the left of the blot. B48* refers to an apoB protein that resembles apoB48 in size. The position of the molecular weight marker myosin (200 KD) is shown on the right.

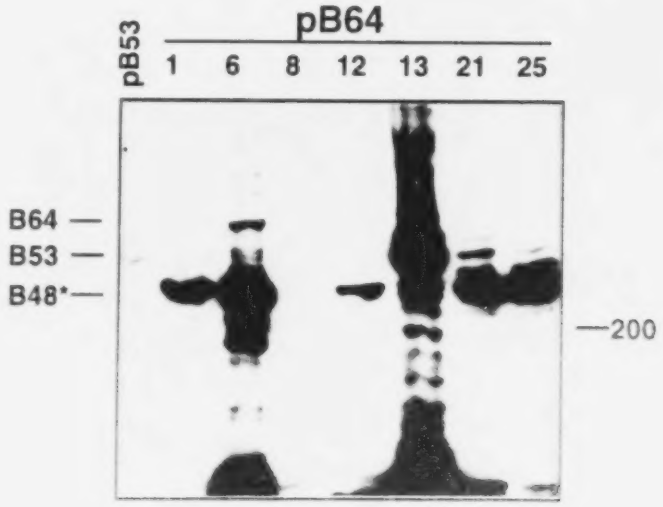


**Figure III.2:** Stable transformants of McA-RH7777 cells that secrete recombinant human apoB72 and apoB100 species. Stably transfected McA-RH7777 cells (50-60 % confluent) were cultured in serum free media for 24 hours. The media was collected and human apoB proteins were detected with antibodies specific to human (A.) or rat (B.) apoB species as described in Figure III.1. Positions of the apoB species with the expected molecular mass are indicated on the left of the blots. B48* refers to an apoB protein that resembles apoB48 in size.

**Figure III.3:** Verification of the carboxyl-terminal epitope of the apoB64 protein produced by stably transfected McA-RH7777 cells. The cells (50-60 % confluent) were cultured in serum free media and processed as described in Figure III.1. Membranes were probed with monoclonal antibodies 3F5, 4G3 or polyclonal antibodies 2140. Media from cells expressing either human apoB48, B53, B60 or B72 were included as controls for the specificity of the antibodies. The epitope position for each antibody is shown on a linear map of apoB100 below the panels.

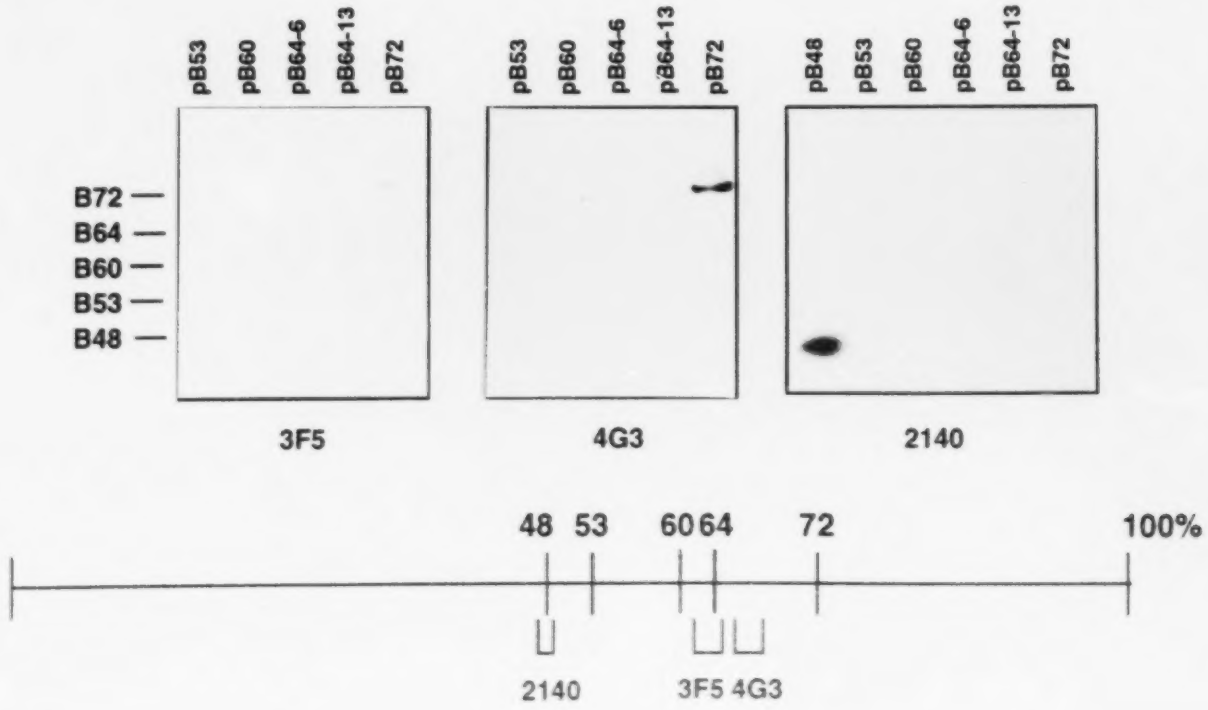


**Figure III.4:** The buoyant density of lipoproteins containing recombinant and endogenous apoB. Stable transformants harbouring truncated apoB64, B72 and the full length apoB100 or nontransfected cells were cultured in serum free media for 24 hours. The conditioned media were collected and subjected to ultracentrifugation on a salt gradient. Twenty fractions of 2 ml each were collected from the bottom of the centrifuge tubes and the apoB proteins in each fraction separated and visualized by immunoblotting with A. antibody specific for human apoB and B. antibody specific for rat apoB.

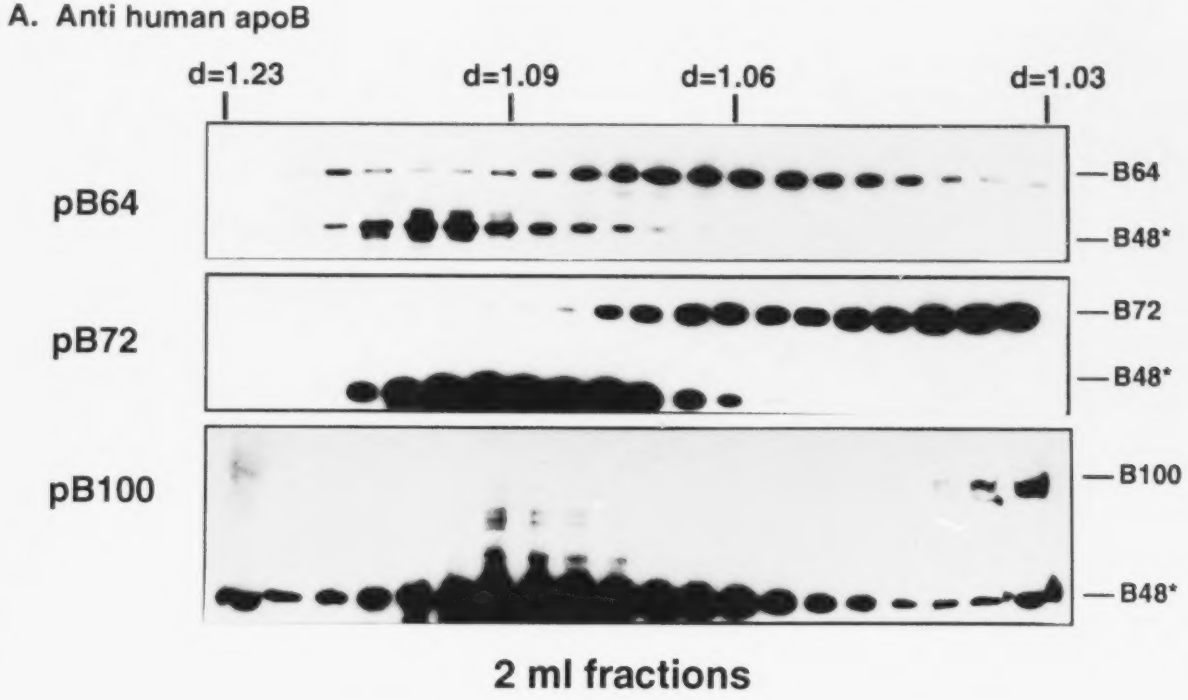


**Figure III.4:** continued

**B. Anti rat apoB**

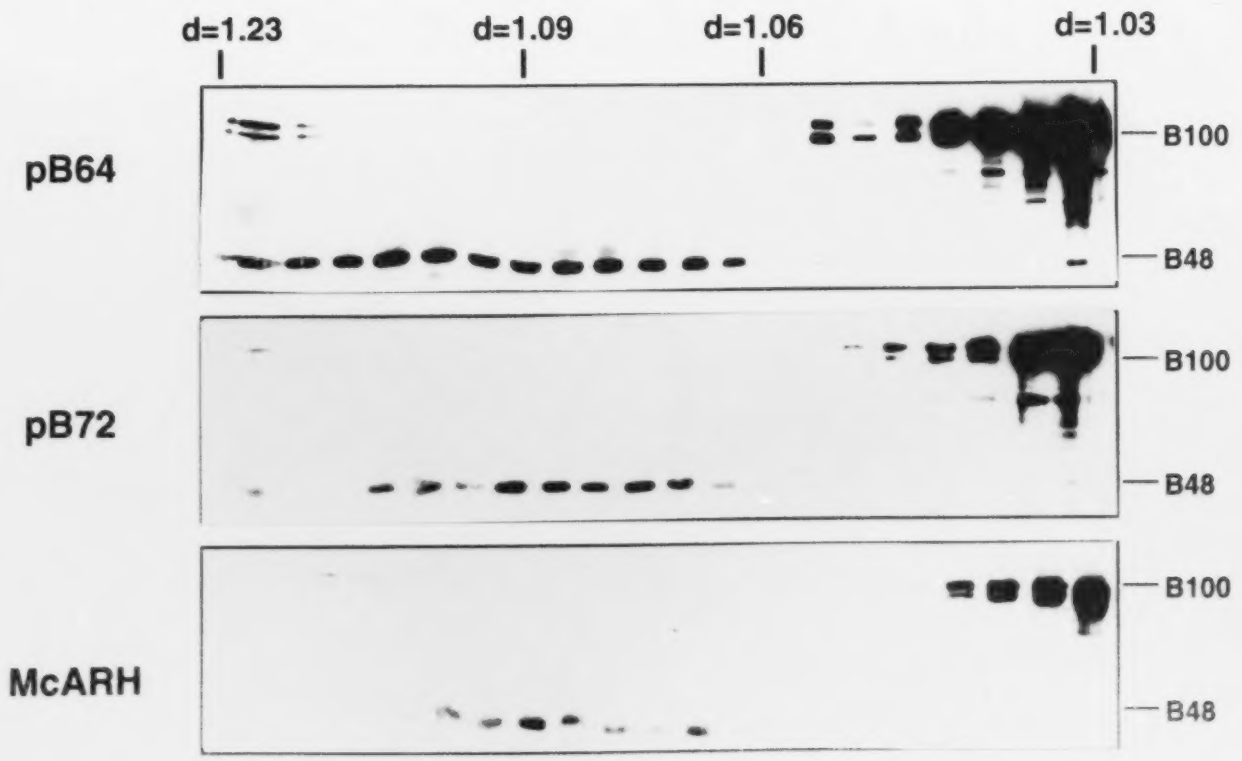


**Figure III.5:** Agarose gel electrophoresis of the secreted lipoproteins. Lipoproteins in the conditioned serum-free medium of apoB72 and apoB100 expressing cell lines were resolved by agarose gel electrophoresis followed by immunoblot analysis with anti-human apoB (A.), anti-rat apoE (B.), anti-rat apoAI (C.) antibodies. Lipoproteins isolated from rat or human plasma (h.LDL, human LDL; r. HDL, rat HDL) were used as standards. Positions of β-, pre-β, and α-migrating lipoproteins are indicated on the right.

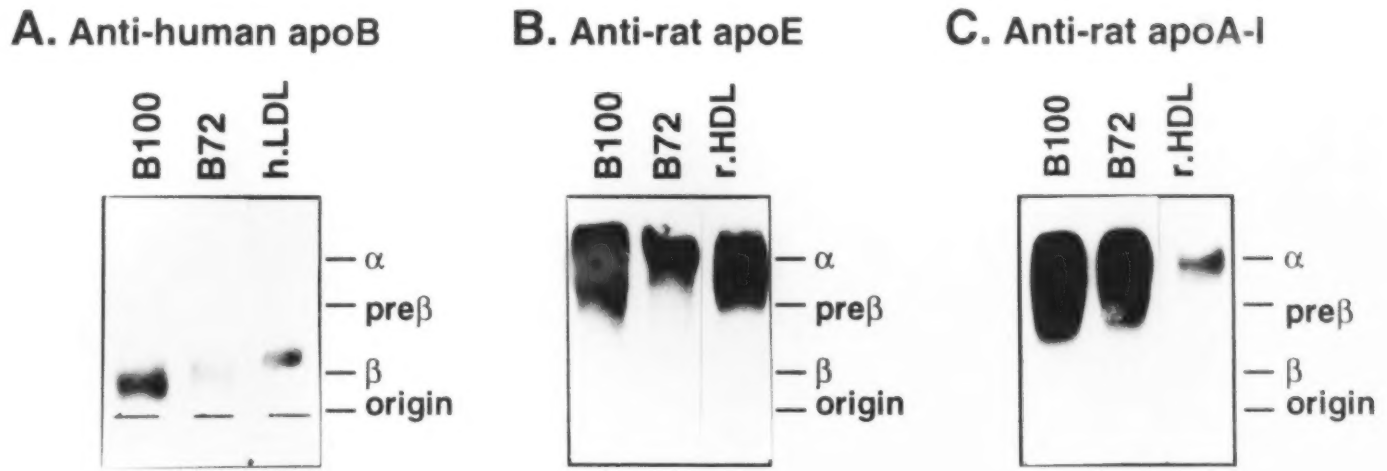


**Figure III.6:** Plot of peak density of truncated apoB-containing lipoproteins *versus* % of apoB100. The densities of all but apoB64-containing lipoproteins are reproduced from Yao *et al.* (1991 and 1992) and McLeod *et al.* (1994). Data for apoB53-, apoB64- and apoB72-containing lipoproteins was obtained from- or verified in- chapter III and IV. With exception of B53-10 (n=1), all density gradient fractionations were performed at least twice with identical results. The positions of the regions predicted to have high amphipathic α helices ( ) and β strand ( ) content are shown above the plot (Segrest *et al.* 1994; discussed in section III.3.2.).

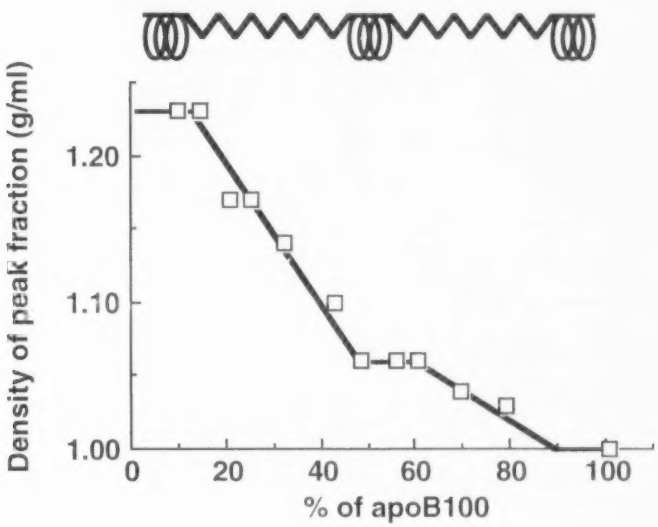


**Figure III.7 - III.9:** Kinetics of synthesis and secretion of recombinant human apoB and rat apoAI and apoB100. Cells were incubated with 1 ml methionine-free DMEM containing 200 μCi [$^{35}$S]methionine for 2 hours. The pulse medium was then replaced with 1.0 ml DMEM and the cells incubated for the indicated time points. At each time point, media were collected and apoB recovered by immunoprecipitation with anti-human apoB antisera which recognizes human and rat apoB. The apoB proteins were resolved by electrophoresis on a 5 % polyacrylamide 0.1 % SDS gel, stained, impregnated with fluor and subjected to fluorography. The amount of radioactivity in the recombinant or endogenous apoB was quantified and plotted against time. ApoAI was immunoprecipitated with anti-human antisera and resolved by electrophoresis on a 10 % polyacrylamide 0.1 % SDS gel and processed as for apoB. *Top and middle panels:* kinetics of cellular (top panel) and medium (middle panel) apolipoprotein; light symbols denote intracellular apolipoprotein, dark symbols, apolipoprotein secreted into the media. Circles indicated data from cell lines B72-38 (Figure III.7), B100-18 (Figure III.8) and B64-17 (Figure III.9); squares, data from cell lines B72-21 (Figure III.7) and B100-24 (Figure III.8). The Stineman routine (CA Cricket Graph) was used so that the fitted line for a given data set passes through each and every point. Half-time values are interpolated as shown. *Bottom:* total apolipoprotein recovered (sum of cell and medium % radiolabel) over time. Dark bars denote data from B72-38 (Figure III.7). B100-18 (Figure III.8), and B64-17 (Figure III.9); light bars, data from B72-21 (Figure III.7) and B100-24 (Figure III.8). The experiments for B72-21 and B100-24 were performed once, whereas experiments for B72-38, B100-18 and B64-17 were performed twice with essentially identical results.

**Figure III.7:**

**A. Human apoB72**

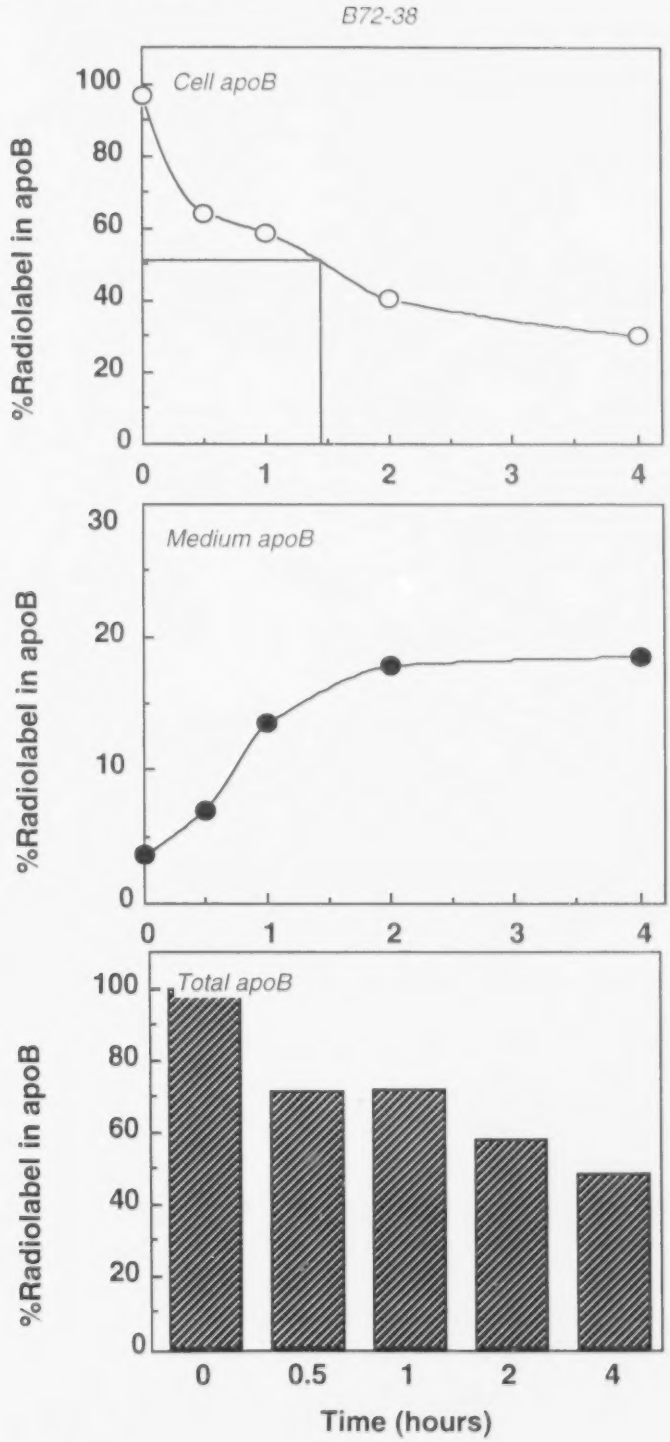


**Figure III.7:** continued

## B. Rat apoB100

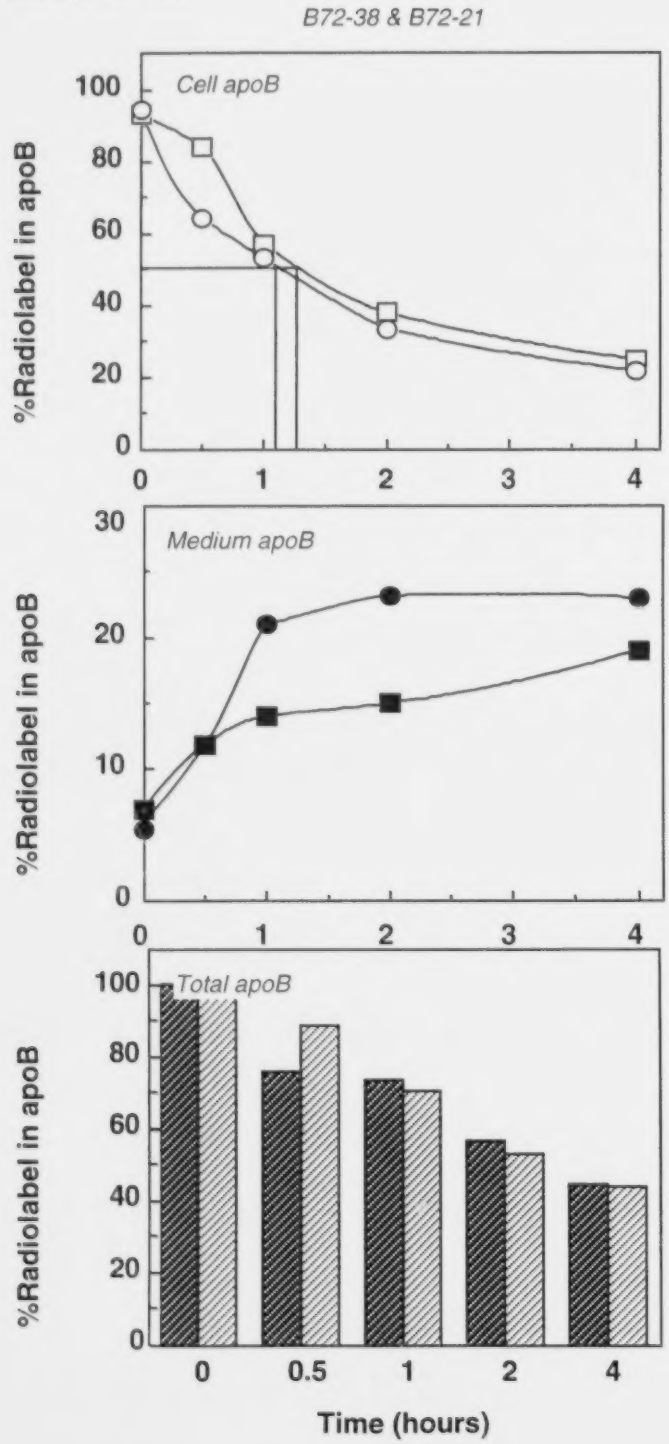


**Figure III.7:** continued

## C. Rat apoAI

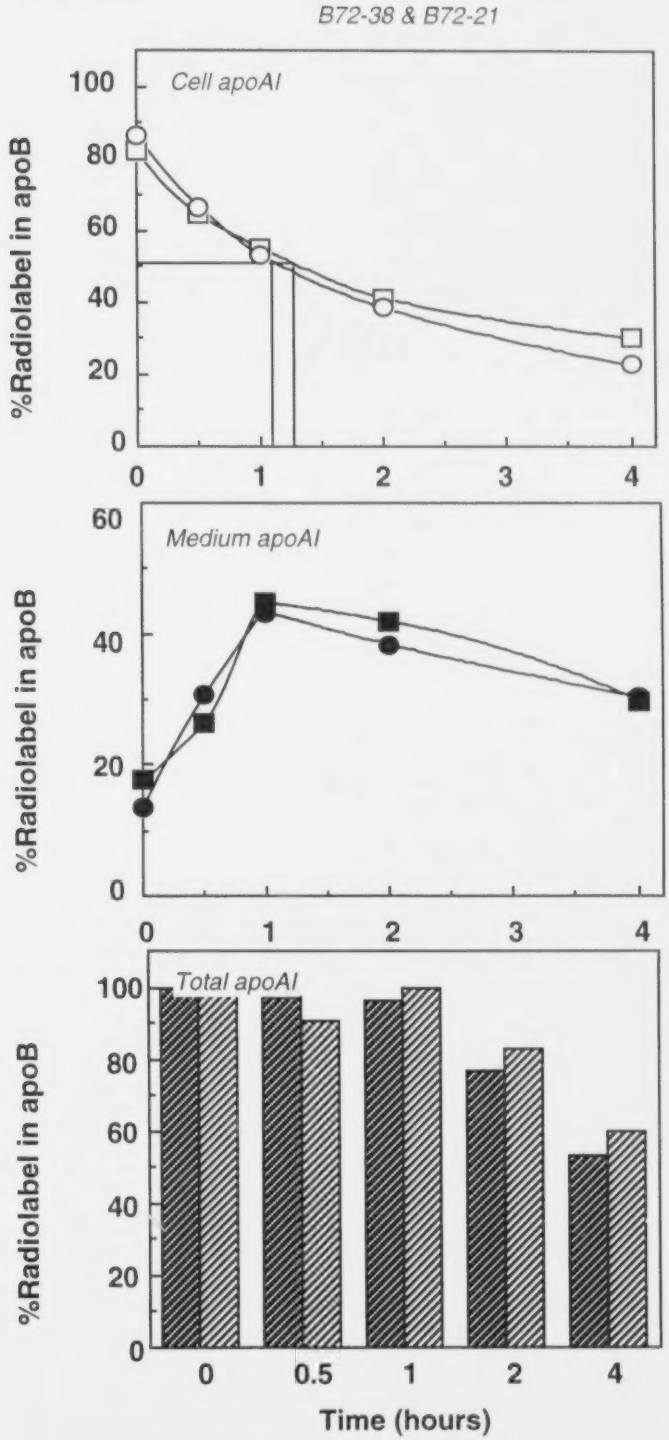


Figure III.8:

## Human and rat apoB100

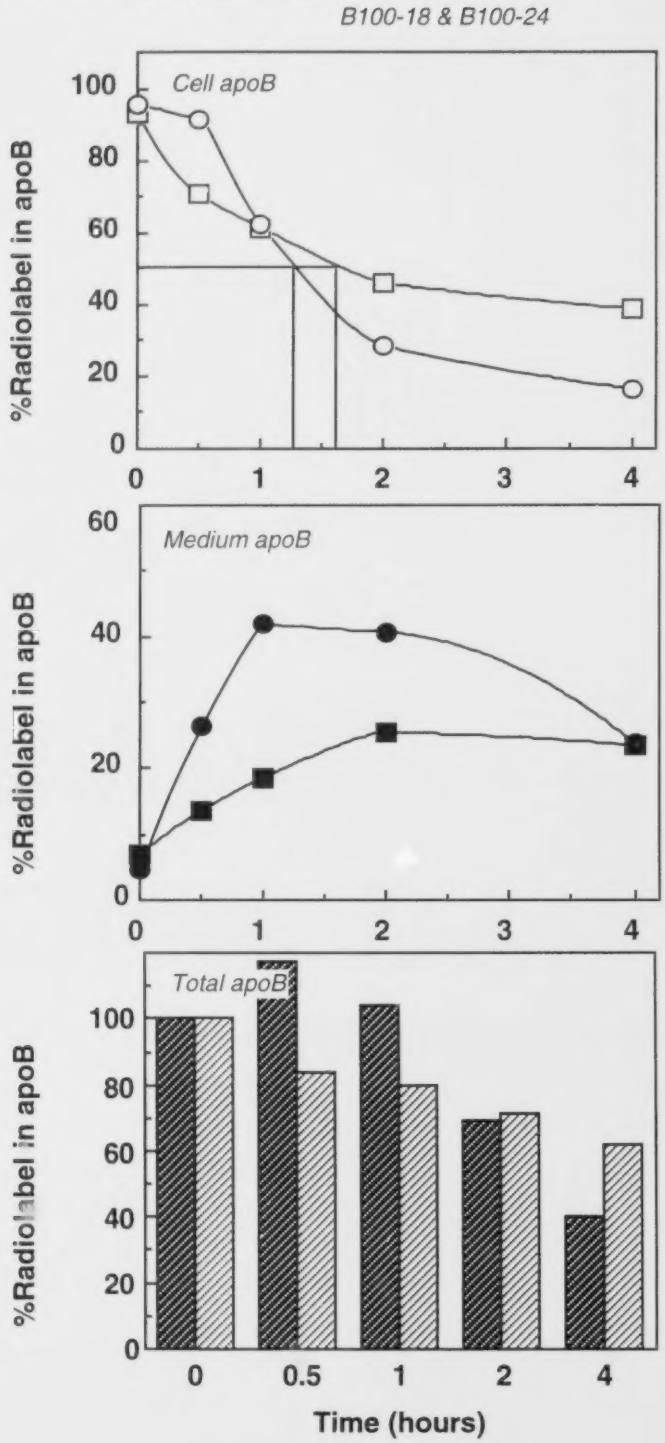


**Figure III.9:**

## A. Human apoB64

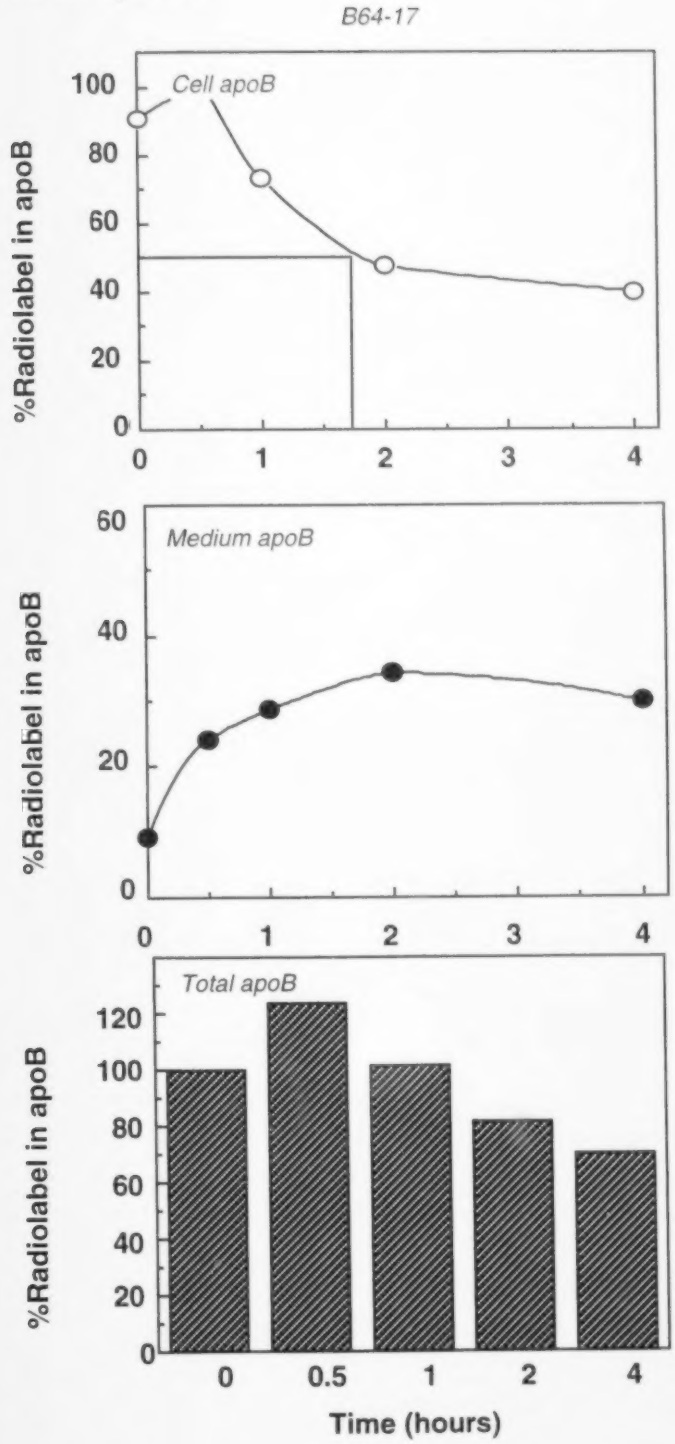
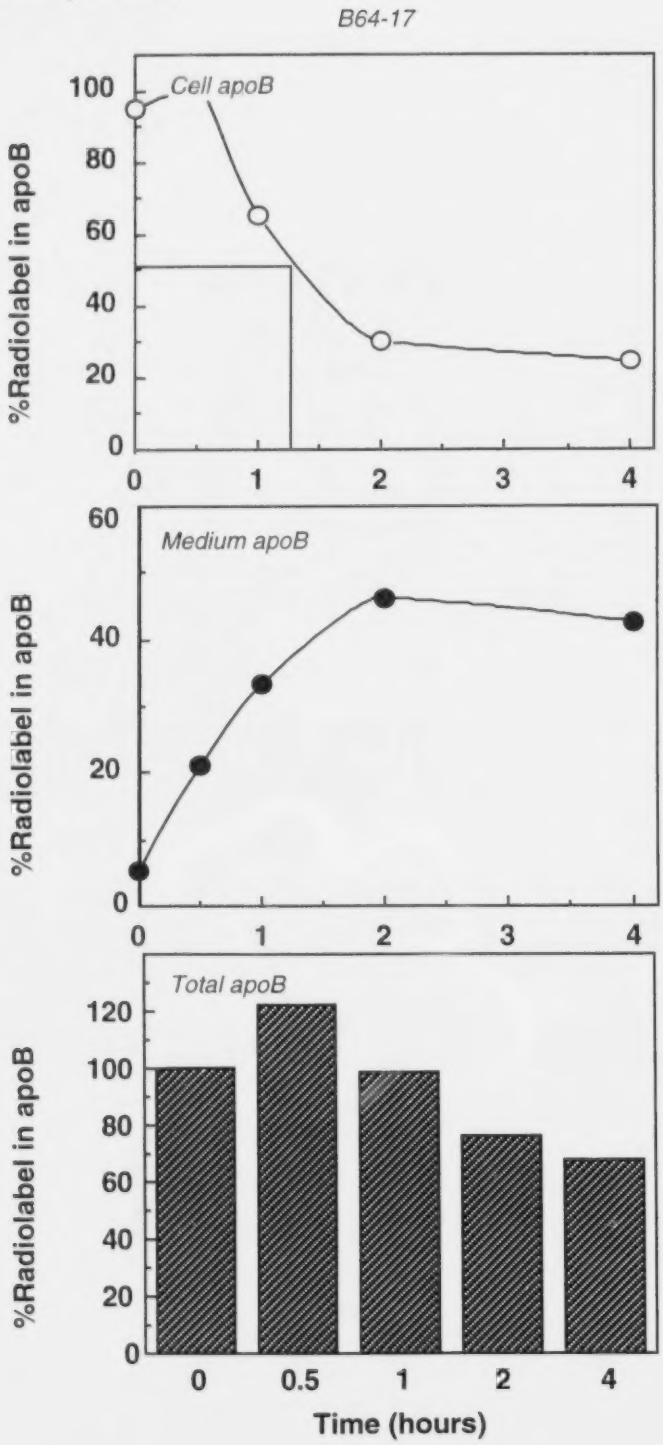


**Figure III.9:** continued

## B. Rat apoB100



# Chapter IV. EFFECT OF OVEREXPRESSION OF APOB ON APOB-CONTAINING LIPOPROTEIN ASSEMBLY AND SECRETION*

## IV.1. INTRODUCTION

Regulation of hepatic apoB100 secretion occurs at multiple levels as outlined in the introduction of this thesis. Briefly, some studies indicate that hepatocytes respond to various stimuli by changing levels of apoB mRNA and apoB100 protein synthesis (Dashti 1992, Zhang *et al.* 1993, Sparks *et al.* 1992, Wu *et al.* 1994). Other studies show that apoB mRNA levels are refractory to change and control of apoB100 secretion occurs post-translationally: newly synthesized apoB100 proteins are targeted for either secretion or intracellular degradation, depending on the conditions of the cell (Pullinger *et al.* 1989). Factors affecting secretion efficiency, *i. e.*, the portion of newly synthesized apoB100 that is secreted rather than degraded, have been the object of intense investigation. Since nascent apoB100-containing lipoproteins consist of triglyceride, free and esterified cholesterol, and phospholipid, conditions affecting synthesis of these lipids have been proposed to regulate apoB secretion in various model systems. For example, substrates for synthesis of lipids such as triglyceride (Borén *et al.* 1993), cholesteryl ester (Cianflone *et al.* 1990), and phosphatidylcholine (Yao and Vance 1988) have been found to augment apoB100 secretion efficiency. Therefore, it is generally believed that secretion of apoB100 is

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* A portion of this chapter is to be published (Selby and Yao 1995. *Atheroscler Thromb Vasc Biol*, in press).

dependent on conditions affecting lipid availability (Dixon and Ginsberg 1993, Sniderman and Cianflone 1993).

Although changes in the level of apoB mRNA and protein synthesis have been observed as indicated above, it is unknown whether changes in the levels of apoB mRNA could have significant effects on production of apoB100-containing lipoproteins. It might be expected that if apoB mRNA levels were to rise dramatically, the extent of apoB100 protein secreted might still be limited by lipid availability, *i. e.*, result in either no change in the levels of apoB100 secreted, diverting the rest for degradation, or result in secretion of increased numbers of smaller, more dense apoB100-containing lipoproteins. There are, in principle, at least two approaches that can be taken to achieve specific alteration of apoB mRNA levels. One approach is to disrupt one allele of the apoB gene to presumably reduce the level of gene product. Targeted gene disruption has been achieved in HepG2 (Farese *et al.* 1992) but the effect of the disruption on lipoprotein production has not been pursued. Alternately, transgenic mice or cell lines that express different steady state levels of apoB mRNA can be generated. Transgenic mice expressing different levels of human apoB have been created (Linton *et al.* 1993b, Callow *et al.* 1993) and a correlation between gene copy number and plasma apoB100 has been suggested. However, it is unclear if plasma levels of human apoB100 found in the mice are due to transgene expression levels, failure to clear the human apoB100-containing lipoproteins, or both.

In this study, McA-RH7777 were used to generate cell lines that express different steady state levels of human apoB53 (representing the amino-terminal 53 % of apoB100) to determine the effects of expressing different levels of apoB mRNA on the synthesis, secretion, and composition of the human- and rat- apoB-containing lipoproteins. Results obtained suggest that the levels of apoB53 mRNA have a profound effect on the level of secretion of apoB53-containing lipoproteins while having minimal effects on endogenous rat apoB100-containing lipoproteins. Moreover, the levels of apoB53 produced may have stimulatory effects on lipogenesis.

## IV.2. RESULTS

### IV.2.1. Generation of stably transfected McA-RH7777 cells expressing recombinant human apoB53.

Stable transformants expressing different levels of apoB53 were generated as described in chapter II. From eighteen G418-resistant colonies selected, three clones were obtained (B53-1, B53-14, and B53-10) that exhibited different steady state intracellular and secreted levels of apoB53 as determined by immunoblot analysis (Figure IV.1 A.). Densitometric analyses of the immunoblot revealed that clones B53-1 and B53-14 had ~8- and ~3- fold, respectively, more intracellular apoB53 than B53-10. Similarly, accumulation of apoB53 in the medium of clones B53-1 and B53-14 was ~12- and ~3- fold, respectively, higher than that of clone B53-10.

Analysis of the steady state levels of apoB53 mRNA in each clone by RNase protection indicated that apoB53 mRNA levels correlated positively with the steady state level of intracellular and secreted apoB53 protein (Figure IV.1 B.). Densitometric measurement of the RNase protected bands indicated that levels of the apoB mRNA in clones B53-1 were ~4-fold higher than apoB53 mRNA levels in B53-14 while B53-10 messenger levels were beneath the limits of detection. Therefore, clones that express different levels of apoB53 mRNA were obtained.

IV.2.2. Effect of expression of different levels of apoB53 on steady state levels of endogenous rat apolipoproteins

Effects of the transgene expression levels on the steady state levels of endogenous rat apoB100, apoB48 and other apolipoproteins were examined (Figure IV.2 A. and B.; see also Figure IV.4). The steady state levels of endogenous rat apoB100 produced by these clones were compared to the levels in the untransfected parental line by immunoblotting using an antibody specific for rat apoB (Figure IV.2 A.). It was found that rat apoB100 was reduced but in a manner independent of transgene expression level. There was very little apoB48 found in the transfected cells, therefore, the effect of overexpression of apoB53 on apoB48 could not be accurately assessed. Total steady state levels of cellular and secreted proteins also did not appear to be affected by the level of expression of apoB53 as determined by SDS PAGE and Coomassie staining (Figure IV.2 B.). Finally, apoAI and apoE appeared variably affected, again, independent of the level of apoB53 expression (see Figure IV.4 anti-apoAI and apoE

panel). These results are the first indication that the level of expression of human apoB53 may exert little influence on the metabolism of endogenous proteins.

IV.2.3. Characterization of lipoproteins produced by the stable transformants expressing human apoB53

Differences in expression levels of the recombinant human apoB53 might have an effect on the density distribution of the apoB-containing lipoproteins produced by the clones. For instance, it might be expected that with increased levels of expression, the cell may reduce the amount of lipid per lipoprotein particle (endogenous and/or recombinant) in order to accommodate. Therefore, the buoyant density of lipoproteins containing the recombinant apoB53 and rat apoB100 was determined by density gradient ultracentrifugation (Figure IV.3 A. and B.). The density distribution of the apoB53-containing lipoproteins secreted by the clones resemble that of HDL, *i. e.*, they display a broad density range with a peak between d=1.06 and d=1.21 g/ml, regardless of the level of expression (Figure IV.3 A.). Notably, although the construct used to generate the clones was designed to eliminate the formation of apoB48 (Yao *et al.* 1992; see chapter II), an apoB48-like species was observed albeit to a lesser extent than apoB53 (B48* in Figure IV.3 A.). The apoB48-like protein probably arises as a result of the generation of a stop codon on the addition of a polyA tail 10 nucleotides downstream of a cryptic polyadenylation signal as discussed in chapter III (Johnson and Innerarity 1993). When the conditioned media from all the apoB53 secreting clones was used to determine the density

distribution of endogenous rat apoB100 it was found that rat apoB100 was mainly confined to lipoproteins of the low density range (d<1.06 g/ml), again, independent of the level of transgene expression (Figure IV.3 B.). This result is the second indication that the level of expression of apoB53 may have little or no impact on endogenous proteins, and particularly rat apoB100 metabolism.

Flotation of the medium lipoproteins confirmed that apoB100-containing lipoproteins produced by these cells were primarily confined to the d<1.05 g/ml range, whereas apoB53-, apoE- and apoAI- containing lipoproteins remained in the d>1.05 g/ml fraction. This technique also enabled crude analysis of lipids apportioned to the specific lipoproteins by high performance liquid chromatography (HPTLC) (see section IV.2.3).

Apolipoproteins present in apoB53-containing lipoproteins were examined by agarose gel electrophoresis followed by immunoblot analysis (Figure IV.5). Lipoproteins containing recombinant apoB53 exhibited b mobility similar to human LDL (Figure IV.5 A.) and endogenous rat apoB100 (Yao *et al.* 1993). The apoB53-containing lipoproteins were essentially free of apoAI and apoE; these apolipoproteins were associated primarily with a migrating particles (Figure IV.5 B. and C.).

In sum, the clones secrete different levels of HDL sized apoB53-containing lipoproteins that are free of apoAI and apoE, and appear to minimally affect the secretion and density distribution of endogenous apolipoprotein-containing lipoproteins.

IV.2.4. Effects of overexpression of apoB53 on lipid mass and lipid synthesis

Since the buoyant density of lipoprotein particles reflects their lipid content, the fact that the high apoB53 expressing cells (B53-1) secrete more lipoproteins containing apoB53 indicates that these cells may also secrete more lipids. To determine if this is indeed the case, the intracellular and medium lipids were examined by HPTLC (Figure IV.6), and the three major lipids (triglyceride, total cholesterol, and phosphatidylcholine) were quantified by enzymatic assay (Table IV.1 Intracellular Lipids). Data summarized in Table IV.1 indicate that there were no significant changes in the intracellular pool size of total cholesterol or phosphatidylcholine. However, the intracellular triglyceride level was 16 % lower in the high expressor (B53-1) than for the intermediate expressor (B53-14) and 25 % lower than the triglyceride levels of the low expressor (B53-10). These results were further confirmed by HPTLC (Figure IV.6 lanes 9 - 11).

Enzymatic analysis of media triglyceride, total cholesterol and phosphatidylcholine was also performed (Table IV.1 Media Lipids). The high expressor (B53-1) secreted ~2.5 times more triglyceride and phosphatidylcholine over a 17-hour period than the low expressor (B53-10), and secreted ~2.2 times more triglyceride than the intermediate expressor (B53-14). The low level of medium lipids secreted by the transfected cells precluded an accurate estimation by the enzymatic assays, therefore medium lipids were analyzed semi-quantitatively by HPTLC. Since the recombinant apoB53-containing lipoproteins could be separated from the endogenous apoB100-containing lipoproteins by equilibrium

ultracentrifugation as demonstrated earlier (Figure IV.4), medium lipids apportioned to apoB53 and apoB100 containing fractions could be determined (Figure IV.6 lanes 3 - 8). The majority of secreted phosphatidylcholine, cholesterol, and triglyceride was found in the d>1.05 g/ml fraction (Figure IV.6 lanes 4, 6, 8). Furthermore, in the d>1.05 g/ml fraction, secretion of the three major lipids was greater for B53-1 than B53-14 and B53-10 (Figure IV.6 lane 4, 6, and 8). In the d<1.05 g/ml fraction, there appeared to be no difference in the lipids associated with endogenous apoB100; the decreased concentration of cholesteryl ester in the d<1.05 g/ml fraction in cell line B53-1 was not reproducibly observed (Figure IV.6 lane 3, 5, and 7). It is likely that the increased lipid levels in the d>1.05 g/ml fraction of the high expressor are attributable to the increased secretion of apoB53-containing lipoproteins since the other apolipoproteins in this fraction (Figure IV.4 anti-AI and anti-E panel) tend to be associated with very little lipid as indicated by density gradient ultracentrifugation performed previously (Tanabe *et al.* 1989, Yao *et al.* 1993).

[$^3$H]glycerol incorporation experiments using the high (B53-1) and low (B53-10) expressor were performed to determine the contribution of glycerolipid synthesis in secretion of human apoB53- and rat apoB100-containing lipoproteins (Figure IV.7). Indeed, incorporation of the radiolabeled precursor into cellular triglyceride was 2 fold greater in the high expressor (B53-1) than for the low (B53-10) while incorporation into phosphatidylcholine was more or less similar during the first two hour labeling period. Total media lipoproteins contained very little [$^3$H] labeled

phosphatidylcholine so as to preclude an accurate estimation of the difference (if present) between the clones (compare y axes of plots in Figure IV.7) however there was 3 fold (at 2 hours) and 4.5 fold (at 3 hours) more labeled triglyceride incorporated into lipoproteins secreted by B53-1 than for B53-10.

Taken together, results of the metabolic labeling experiments are consistent with the mass measurement of the medium lipid levels.

IV.2.5. Kinetics of synthesis and secretion of apoB species produced by the stably transformed cells

Incorporation of [$^{35}$S]methionine into B53-1 demonstrated a linear relationship from 10 to 30 minutes for apoB53 and from 5 to 30 minutes for apoB100 (Figure IV.8). Therefore, the radioactivity associated with apoB at the end of 30 minute labeling was used as a measure of apoB synthesis. By comparing the intermediate (B53-14) and high (B53-1) expressor it was found that the rate of apoB53 synthesis for B53-1 was ~8 fold higher than B53-14 (84,300 *versus* 10,700 cpm/30 min/mg cell protein). This increased synthesis may account for the 3 fold and 4 fold increase in cell and media apoB53 mass, respectively, observed in clone B53-1 relative to B53-14 (Figure IV.1 A.). However, incorporation into endogenous apoB100 was similar for the high and intermediate expressor (31,360 *versus* 29,980 cpm/30 min/mg cell protein), in agreement with the results obtained with immunoblot analyses above (Figure IV.2 A.).

Pulse chase analysis was performed to examine if there were any changes in the fate of newly synthesized apoB53 and endogenous apoB100 brought on by different levels of expression (Figure IV.9). The intracellular retention of apoB53 for both clones resembled that of apoB48 with a $t_{1/2}$ of ~2.0 and ~3.75 hours for B53-1 and B53-14, respectively (Figure IV.9 A.). Even though the retention time for B53-14 was longer, the secretion efficiency of apoB53 in clone B53-1 was 2 fold greater than B53-14 (38 % *versus* 17 %, respectively; calculated from the values at maximum labeling and the 4 hour time points), similar to the results observed with clones expressing different levels of human apoB100 (see chapter III.). Furthermore, the secretion efficiency of endogenous apoB100 was similar between clones B53-1 and B53-14 (13 % *versus* 8 %, respectively; Figure IV.9 B.), indicating that the level of apoB53 expression minimally affects metabolism of endogenous apoB100. The secretion efficiency of endogenous apoAI was also unaffected by the level of apoB53 expression (Figure IV.9 C.). Approximately 35 % of total labeled apoAI was secreted into the media by 2 hours into the chase, similar to results observed previously (Yao *et al.* 1991, McLeod *et al.* 1994, see also chapter III.). Finally, these results could not be accounted for by reuptake of the lipoproteins produced. A control experiment was performed in which the conditioned media containing the labeled apolipoproteins were added to fresh cells. The data obtained from this experiment (Figure IV.10) indicate that within a 4-hour period, reuptake of the labeled apoB or apoAI by cells was negligible.

Therefore, synthesis and secretion efficiency of apoB53 positively correlates with the level of apoB53 transgene mRNA and the data presented above suggest that endogenous apoB synthesis and secretion might not be affected by apoB53 expression in the transfected cells.

## IV.3. DISCUSSION

### IV.3.1. Summary

While a number of studies have indicated that the level of apoB mRNA remain constant under conditions where the level of apoB100 secretion changes considerably, recent evidence has amassed to suggest that the steady state levels of apoB mRNA can be changed, and the altered apoB mRNA levels may correlate to the level of apoB100 secretion (Dashti 1992, Zhang *et al.* 1993, Wu *et al.* 1994). In the current study, three cell lines stably expressing different levels of human apoB53 were used to assess the interrelationship between the secretion of apoB-containing lipoproteins and the level of apoB gene expression. The results indicate that increased levels of apoB mRNA stimulate apoB protein synthesis and assembly and secretion of apoB-containing lipoproteins. This conclusion is based on methionine incorporation into apoB53 and secretion efficiency as determined by pulse chase analyses in the high expressor *versus* the intermediate (Figure IV.9). Furthermore, the density of the apoB53-containing lipoproteins was unaffected by expression levels (Figure IV.3) suggesting that lipid availability was not limiting. This conclusion was further supported by lipid analysis: increased media lipid mass was observed in

the high expressor (Table IV.1 and Figure IV.6). Unexpectedly, increased incorporation of glycerol into triglyceride in the high expressor relative to the low expressor was observed. Finally, the effects of expression level were most likely not at the expense of endogenous rat apoB synthesis or secretion (Figure IV.2 and IV.9), nor were the density of the endogenous lipoproteins secreted compromised (Figure IV.3).

IV.3.2. ApoB secretion is not limited by lipid availability

The delivery of lipid substrates can stimulate assembly and secretion of nascent apoB lipoproteins (Dixon and Ginsberg 1993). The primary mechanism appears to be post-translational although some evidence of pre-translational control has been observed (Wu *et al.* 1994, Dashti 1992). The current study, however, suggests that apoB may not be subject to passive control by lipid. That is, when apoB expression levels are increased, more apoB is secreted. If apoB secretion was strictly limited by lipid availability it would be expected that only a constant amount of apoB could be secreted while the rest diverted to degradation or, if secreted, have a reduced lipid core to compensate. The present results do not support this view. Instead, neither secretion of rat nor human apoB-containing lipoproteins is compromised, and the apoB-containing lipoproteins secreted into the medium maintain an apoB length-lipoprotein density relationship as observed previously (Yao *et al.* 1991, Spring *et al.* 1992a and b, Graham *et al.* 1991, McLeod *et al.* 1994, chapter III). Furthermore, [$^{3}$H]glycerol incorporation experiments suggest that triglyceride synthesis may be stimulated in cells expressing high levels of apoB53 (Figure IV.7). An

increase in triglyceride synthesis, however, was not matched by a corresponding increase in cellular triglyceride levels in cells expressing high levels of apoB53, and in fact there was a 25 % reduction in cellular triglyceride in these cells relative to the low expressor (Table IV.1). This reduction may in part be due to mobilization of triglyceride stores for lipoprotein assembly and secretion when apoB53 is overexpressed. However, since the rate of [$^3$H] glycerol uptake by the cells and the pool size of intermediates such as glycerol-3-phosphate were not compared between cell lines, it can not be concluded with certainty that overexpression of apoB results in upregulated triglyceride synthesis. The relationship between hepatic apoB synthesis and lipogenesis has not been observed previously and merits further study.

IV.3.3. Secretion of endogenous rat apoB100 is not affected by the level of expression of human apoB53

Overexpression of recombinant human apo48 in McA-RH7777 cells decreased the secretion of endogenous rat apoB100 (Hussain *et al.* 1995). Decreased secretion was due to decreased synthesis of rat apoB100 at steady state but not due to decreased transcription of the gene nor due to a decrease in secretion efficiency. In the current study, a reduction in steady state levels of rat apoB100 was observed in human apoB53 expressing cells. However, expression of human apoB53 did not seem to influence synthesis of rat apoB100 nor was the reduction dependent on level of expression of the transgene. The steady state levels of total protein were unaffected (Figure IV.2 B.) as were apoAI and apoE (Figure IV.3 C.) in the

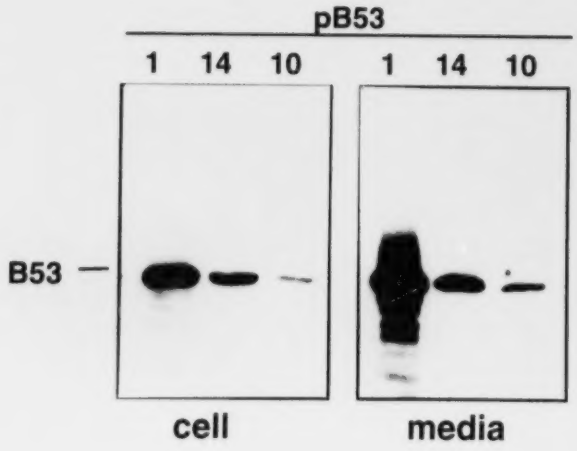
human apoB53 expressing cells, suggesting that overall protein metabolism was unaffected. Furthermore, the effect could not be attributed to overall impairment of secretion since the secretion efficiency of apoAI in both the intermediate and high expressor resembled that of the untransfected parent line (Figure IV.9; McLeod *et al.* 1994). The secretion efficiency of rat apoB100 may be reduced in the intermediate and high expressor relative to nontransfected cells. However, the effect might be clone specific rather than expression level dependent since the secretion efficiency of rat apoB100 in the intermediate expressor was less than that of the high expressor. Although the synthesis and secretion efficiency of rat apoB100 could not be distinguished from human apoB100 in cell lines expressing different levels of human apoB100 (see chapter III), the reduction in steady state levels of rat apoB100 relative to nontransfected cells was not observed nor was it observed for expression of apoB64 and apoB72 (see chapter III). It could be possible that the level of human apoB100 expression (or that of the other cell lines expressing human carboxyl truncated apoB longer than 53 % of the full length protein) did not approach that of human apoB53 (or human apoB48 in the previous study, for that matter) so as to exert this effect. This possibility seems unlikely though since the reduction in endogenous apoB100 was apparent even in the low apoB53 expressor. Alternately, as suggested in chapter III, the effect may reflect an apoB length dependence much like that seen with the buoyant density of apoB-containing lipoproteins or secretion kinetics (McLeod *et al.* 1994, McLeod and Yao 1994).

IV.3.4. Physiologic relevance

Although the results of the current study suggest that vastly different apoB mRNA levels may influence apoB synthesis and secretion, the *in vitro* model utilized here may not represent the *in vivo* situation. At present, no metabolic perturbation has resulted in changes in hepatic apoB mRNA levels by more than 50 %. However, the current results may have bearing on the results observed in mice expressing human apoB (Linton *et al.* 1993b; Callow *et al.* 1994). In these models a rough correlation between human apoB transgene copy numbers and plasma apoB was observed. The high levels of human apoB100 probably arise as a result of a combination of a high rate of human apoB100 synthesis and/or a reduced rate of catabolism (human apoB100 is poorly recognized by the mouse LDL receptor) (Young *et al.* 1994; references cited within). Although not rigorously tested, high transgene copy numbers would most likely lead to high levels of human apoB mRNA, protein synthesis and secretion (Young *et al.* 1994) as observed in the current study. Notably, in these animals, overexpression of human apoB did not lead to a reduction in mouse plasma apoB (Linton *et al.* 1993b, Callow *et al.* 1994). While this may be due to sequestration of apoE or other factors by the human apoB-containing lipoproteins that could reduce the ability to clear the mouse protein, it is also possible that, as suggested by the current study, the level of apoB secretion may not be passively controlled by lipid availability such that the production of the mouse apoB is not curtailed. Thus, the present data could be compatible with transgenic mice studies.

**Figure IV.1:** Generation of stably transfected McA-RH7777 cells expressing recombinant human apoB53. A. Immunoblots of human apoB53. Cells were incubated with serum-free media for 24 hours . Aliquots of the cell (left panel) or medium (right panel) samples derived from equal amounts of cell protein were separated by SDS PAGE (5 % gel) followed by immunoblotting using monoclonal 1D1. B. RNase protection assay of the apoB53 mRNA. Total RNA (15 µg) from the stably transfected cells or non-transfected McA-RH7777 (MH) and HepG2 were hybridized with a $^{32}$P-labeled antisense probe and then digested wtih ribonuclease T1. The protected RNA sequences were resolved on an 8 % sequencing gel and detected by autoradiography. The length of protected apoB mRNA fragments from the transformed cell lines is shorter than that of HepG2 since the transgene lacks nucleotides 1-15 of the apoB cDNA which the probe encompasses.

**A. Anti-human apoB**




**B. RNase protection**

**Figure IV.2:** Effects of transfection on endogenous levels of apolipoproteins. A. Detection of endogenous apoB. Aliquots of cell and media proteins were resolved by SDS PAGE and transfered to nitrocellulose membranes as described in Figure IV.1. The membrane was then probed with polyclonal antibodies specific for rat apoB. B. Detection of endogenous apolipoproteins. Aliquots of cell and media proteins were resolved on a 3 - 20 % gradient gel and stained with Coomassie R250. B100, apoB100; B48, apoB48; alb, albumin; E, apoE; A, apoAI. The identities of apoE and apoAI were confirmed by immunoblot analyses (see Figure IV.4).

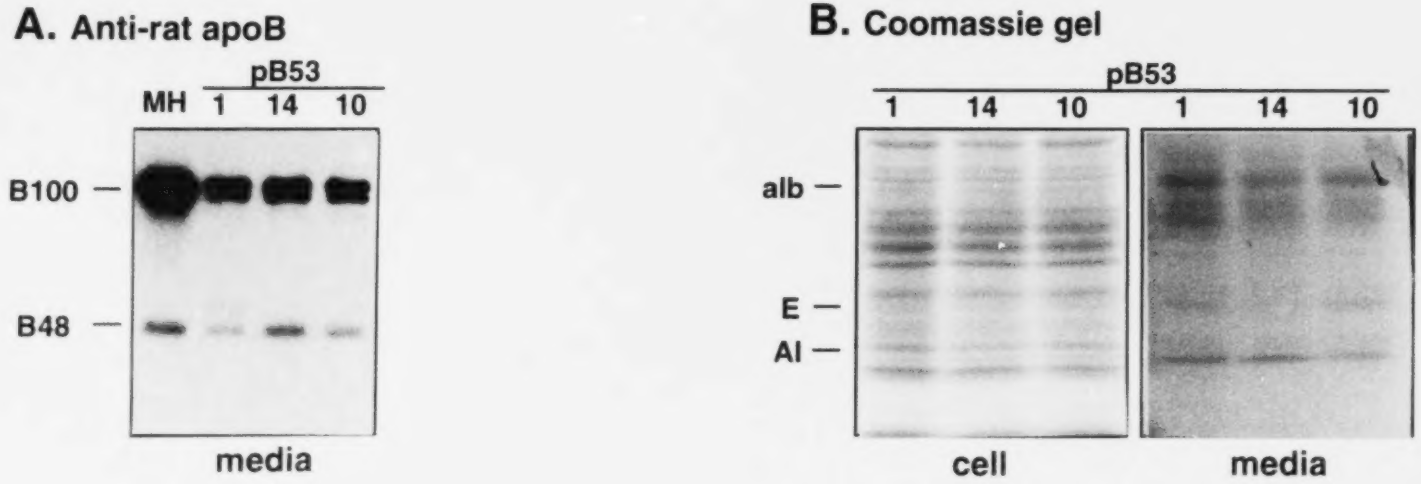


**Figure IV.3:** Density distribution of lipoproteins containing recombinant human apoB53 or endogenous rat apoB100. Cells were incubated with serum-free media (8.5 ml/100 mm dish) for 24 hours. The conditioned media from two dishes were combined and fractionated over a density gradient ranging from d=1.23 to d=1.03 g/ml. Proteins in each fraction were resolved by SDS PAGE (5 % gel) followed by immunoblotting. Human apoB53 (A.) was detected with monoclonal antibody 1D1. The bands in the high density fractions (denoted with an X) are proteins that nonspecifically bind the secondary antibody used in the detection system. Endogenous rat apoB100 (B.) was probed with a polyclonal antibody specific for rat apoB. Occasionally apoB100 was observed in the bottom fraction of the gradient (*e. g.* B53-14, d=1.23 g/ml fraction).

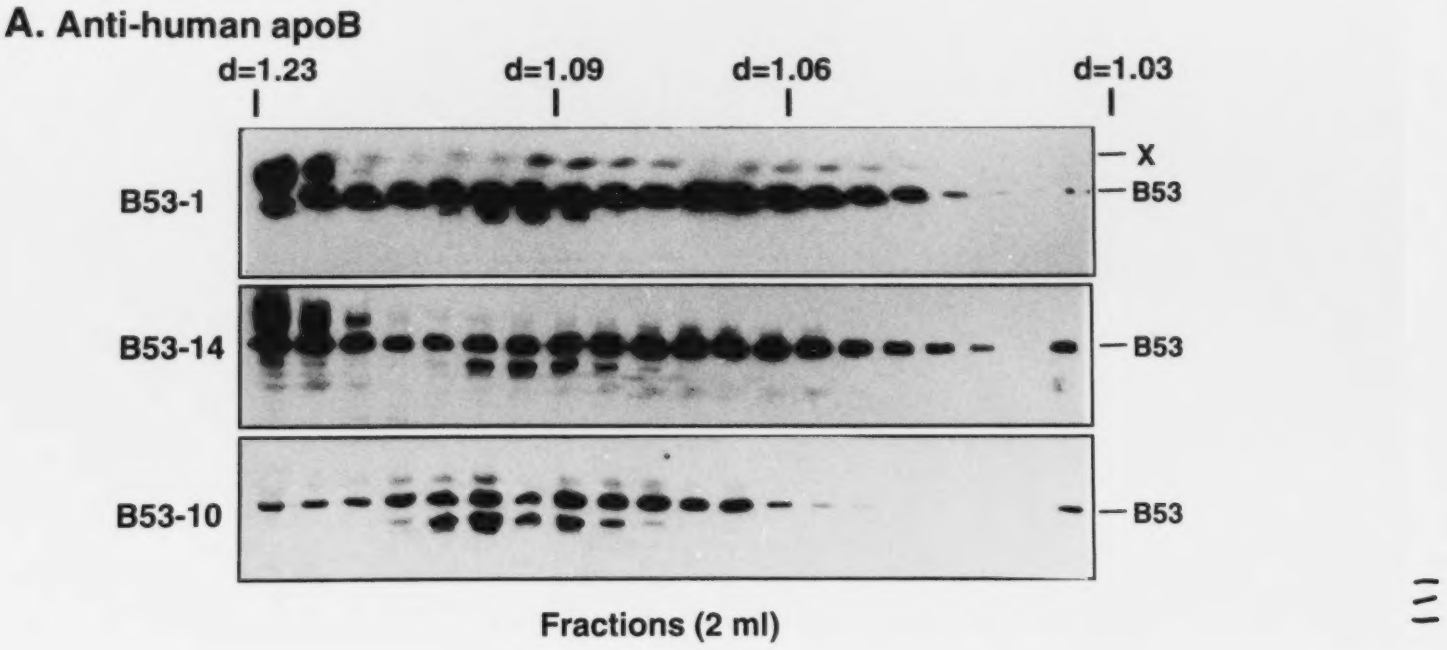


**Figure IV.3:** continued

## B. Anti-rat apoB

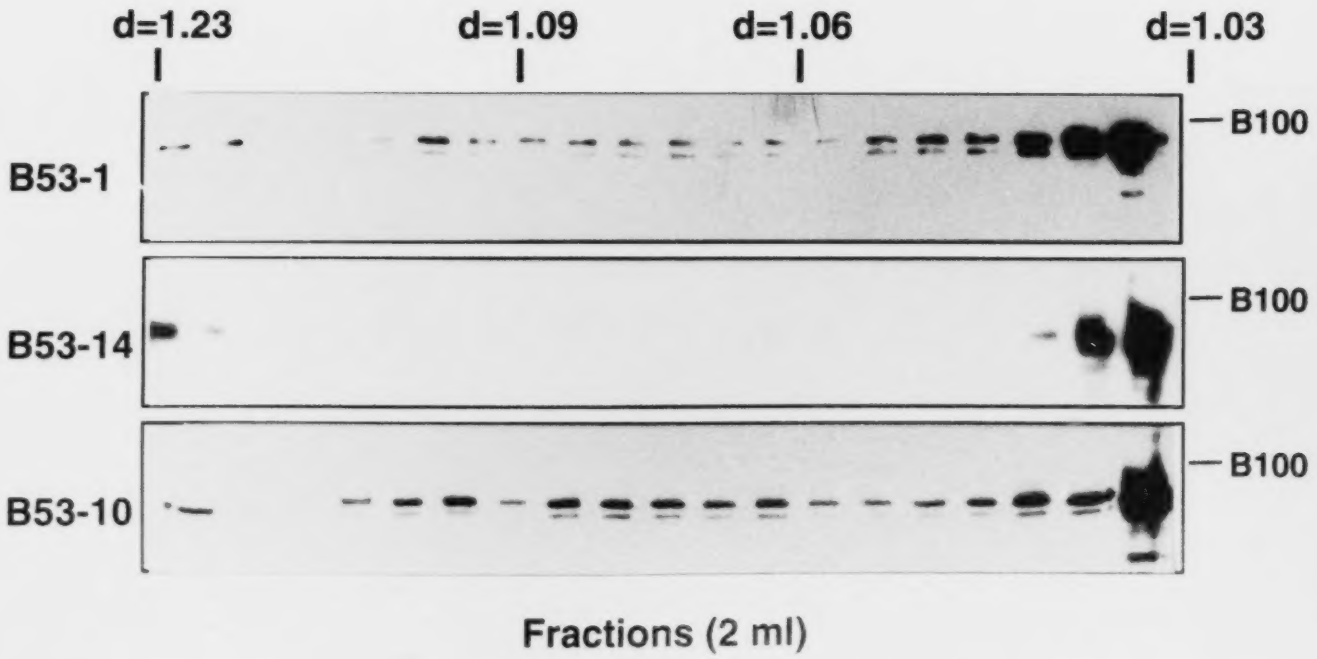


**Figure IV.4:** Separation of endogenous and recombinant media apolipoproteins by single spin ultracentrifugation. Cells from 4 60 mm dishes were incubated in serum free DMEM for 24 hours and the media combined with potassium bromide to achieve d=1.05 g/ml and centrifuged for 24 hours at 50000 rpm 10$^{o}$C . Two fractions were collected and the proteins resolved by SDS PAGE followed by immunoblotting. Human apoB53 (A.), rat apoB100 (B.), and rat apoAI and apoE (C.) were detected as described in chapter II.

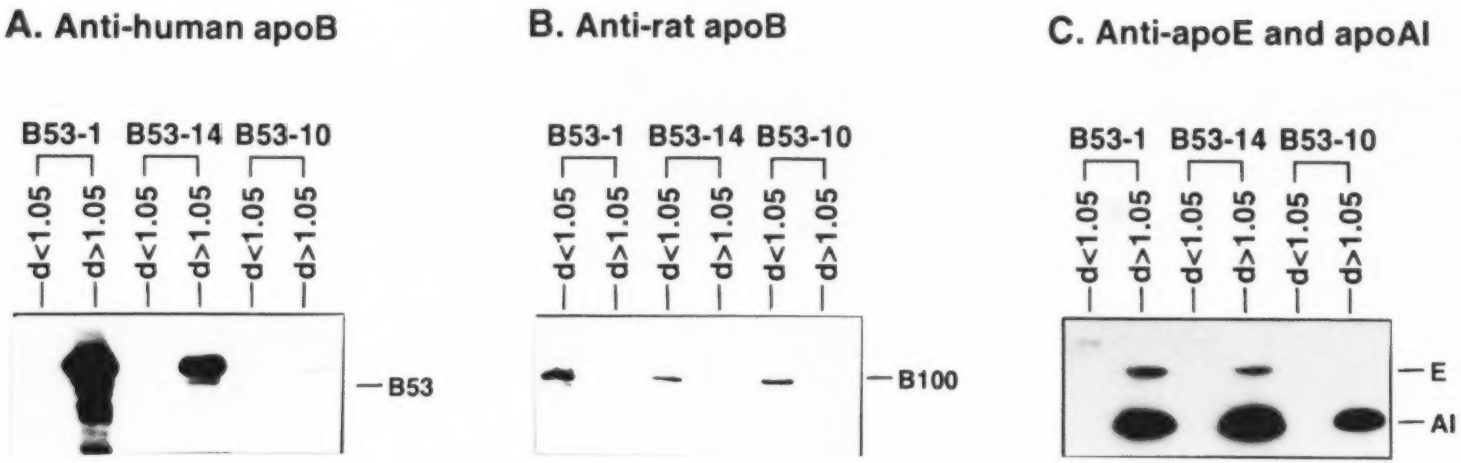


**Figure IV.5:** Agarose gel electrophoresis of the secreted lipoproteins. Lipoproteins in the conditioned serum-free medium of the high apoB53-expression cell line (B53-1) were resolved by agarose gel electrophoresis followed by immunoblot analysis with anti-human apoB (A.), anti-rat apoE (B.), anti-rat apoAI (C.) antibodies. Lipoproteins isolated from rat or human plasma (h.LDL, human LDL; r. HDL, rat HDL) and conditioned medium of stable McA-RH7777 cell line expressing human apoB100 (B100) were used as standards. Positions of β-, pre-β, and α-migrating lipoproteins are indicated on the right.

**Figure IV.6:** Separation of cell and medium lipids. Media from four 60 mm dishes of cells were pooled and fractionated by single spin ultracentrifugation. Lipids from one dish of cells and the media fractions were extracted. Aliquots of medium or cell lipid samples derived from equal amounts of cell protein were resolved by hptlc as described in chapter II. Lipid standards (std) are indicated on the right: CE, cholesteryl ester; TG, triglyceride; FA, fatty acid; FC, free cholesterol; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPM, sphingomyelin.

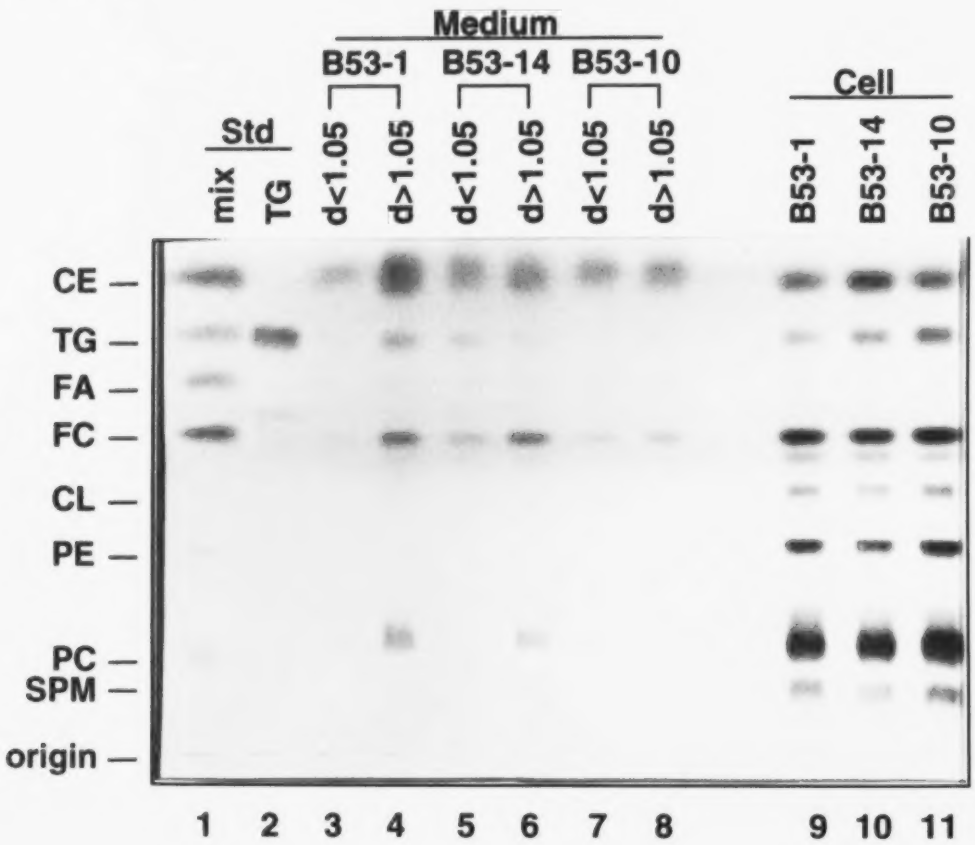


**Figure IV.7:** Incorporation of [$^3$H]glycerol into triglyceride and phosphatidylcholine. Cells (60 mm) expressing high (B53-1, open squares) or low (B53-10, closed squares) levels of apoB53 were incubated with 1 ml serum-free medium containing 10 μCi [$^3$H]glycerol. At the indicated time points, lipids were extracted from cells (left panels) or media (right panels) and resolved by thin layer chromatography. Shown are the results of a single experiment. A similar result was obtained when cell lines expressing high and low levels of human apoB100 were used (Selby and Yao 1995). $^3$H-TG, $^3$H-labeled triglyceride; $^3$H-PC, $^3$H-phosphatidylcholine.

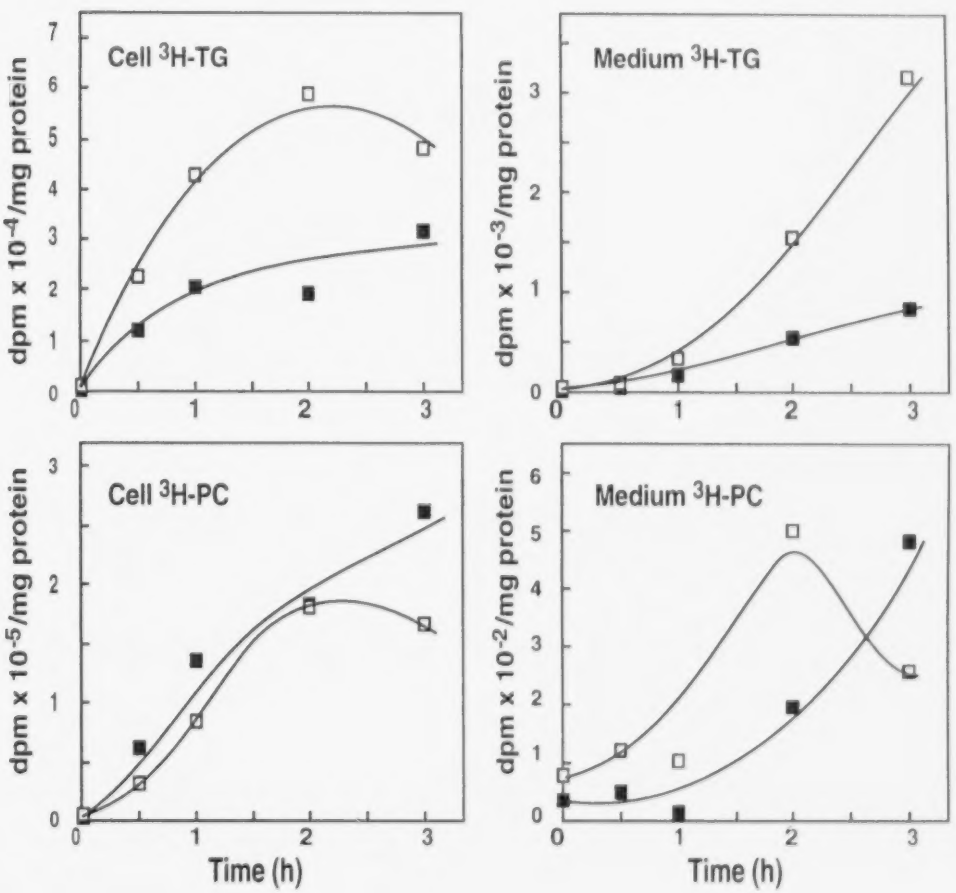


**Figure IV.8:** Incorporation of [$^{35}$S]methionine into cellular recombinant and endogenous apoB. Cells (clone B53-1) were incubated with methionine-free DMEM containing 200 µCi [$^{35}$S]methionine. At the indicated time points, cell apoB proteins were immunoprecipitated with an anti-apoB antiserum which recognises both human and rat apoB. The amount of radioactivity associated with apoB53 (open squares) or endogenous apoB100 (open circles) was quantified. Inset, fluorogram of the $^{35}$S-labeled apoB proteins resolved by SDS PAGE (5 % gel). A second experiment yielded similar results.

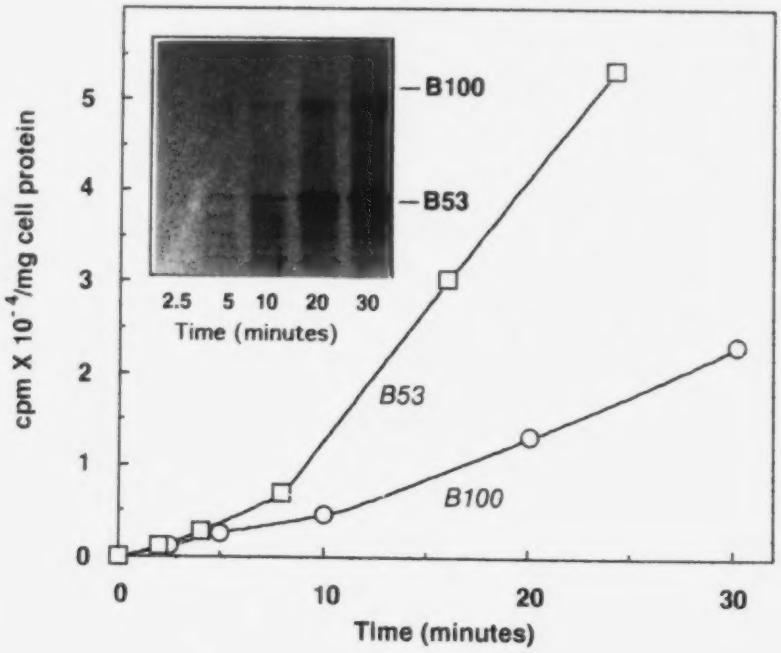


**Figure IV.9:** Pulse chase analyses of cells expressing intermediate (B53-14) and high (B53-1) levels of human apoB53. Cells were incubated with 1 ml methionine-free DMEM containing 200 μCi of [$^{35}$S]methionine for 30 minutes. The pulse media was then replaced with 1.5 ml DMEM supplemented with 2 mM methionine and apolipoprotens recovered and processed as described in chapter II and chapter III (Figure III.7 - III.9). *Top panels:* cellular apolipoproteins. *Middle panels:* media apolipoproteins. *Bottom panels:* total apolipoproteins. Circles, dark bars: B53-1. Squares, light bars: B53-14.

**Figure IV.9:**

**A. Human apoB53**

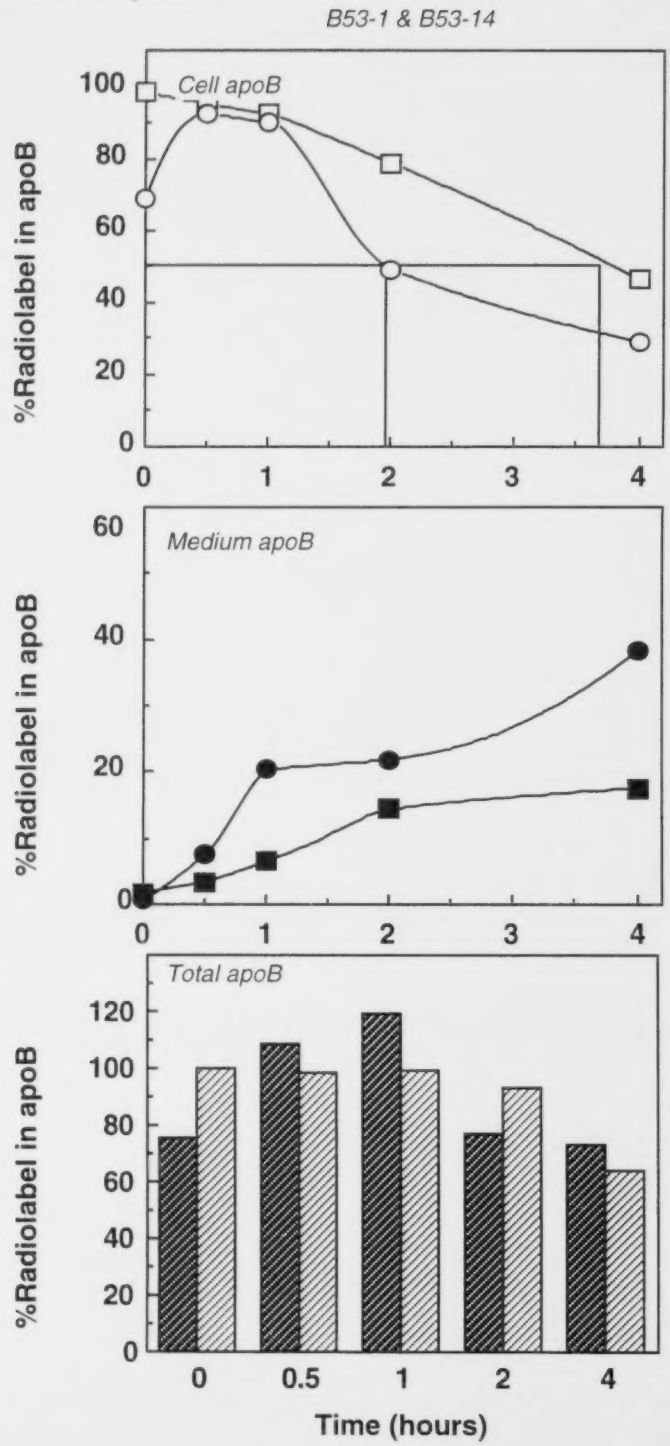


**Figure IV.9:** continued

## B. Rat apoB100

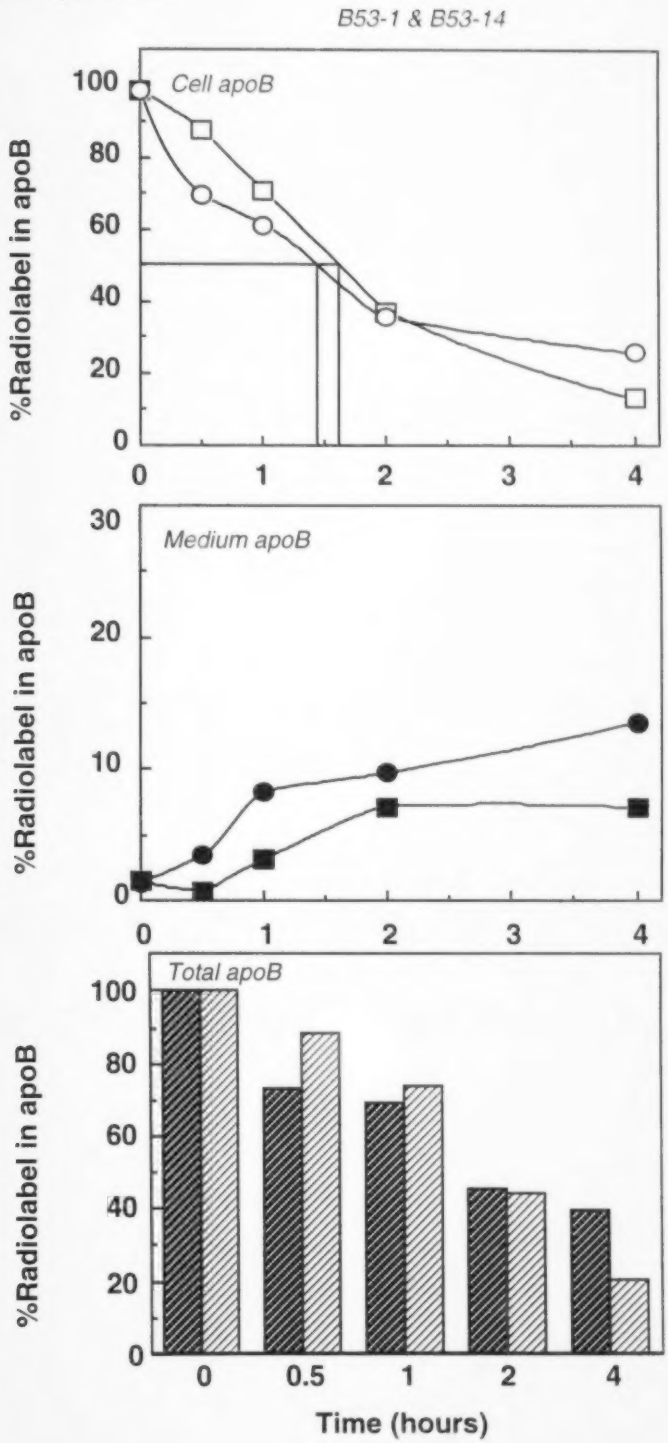


**Figure IV.9:** continued

## C. Rat apoAI

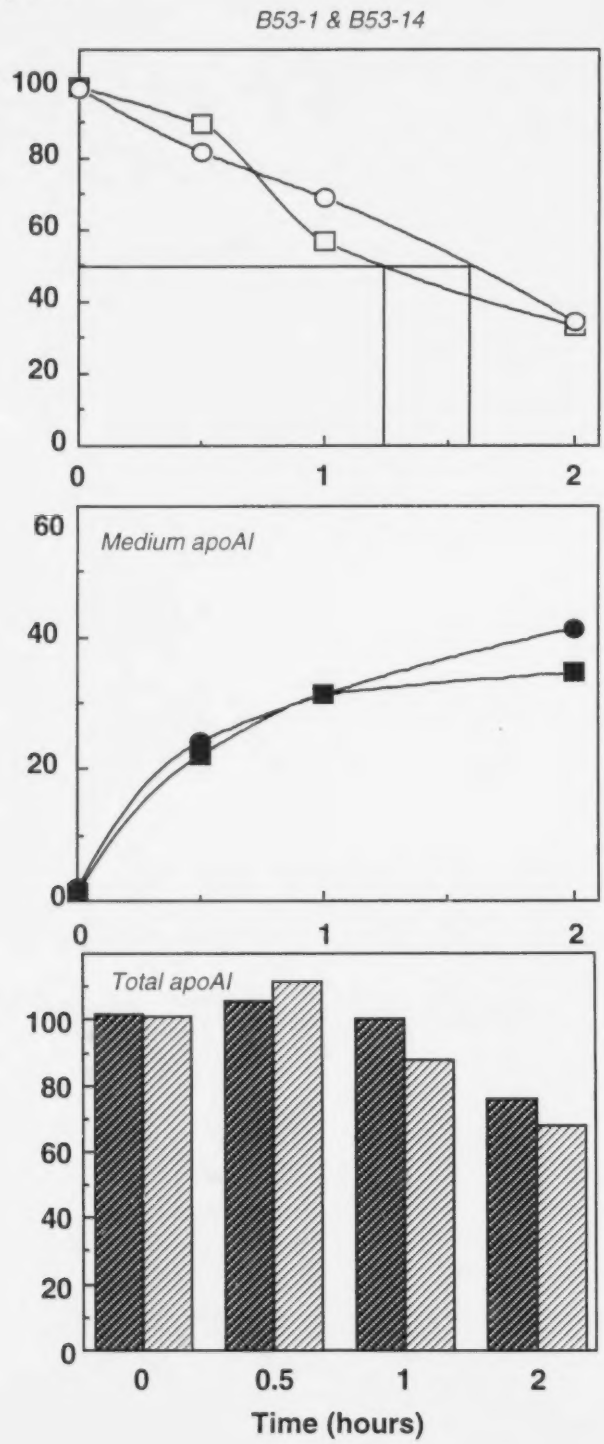


**Figure IV.10:** Association of pre-labeled apolipoproteins with cells. Cells (clone B53-1, 10 cm dish) were labeled with 2.2 mCi [$^{35}$S]methionine for 2 hours and then incubated with DMEM supplemented with 2 mmol/l methionine for 2 hours (chase). The chase media containing labeled apolipoproteins were added to fresh cells. At the indicated times, apoB and apoAI were precipitated from cells (open bars) and media (dark bars) and the radioactivity quantified as described in chapter II. A second experiment yielded similar results.

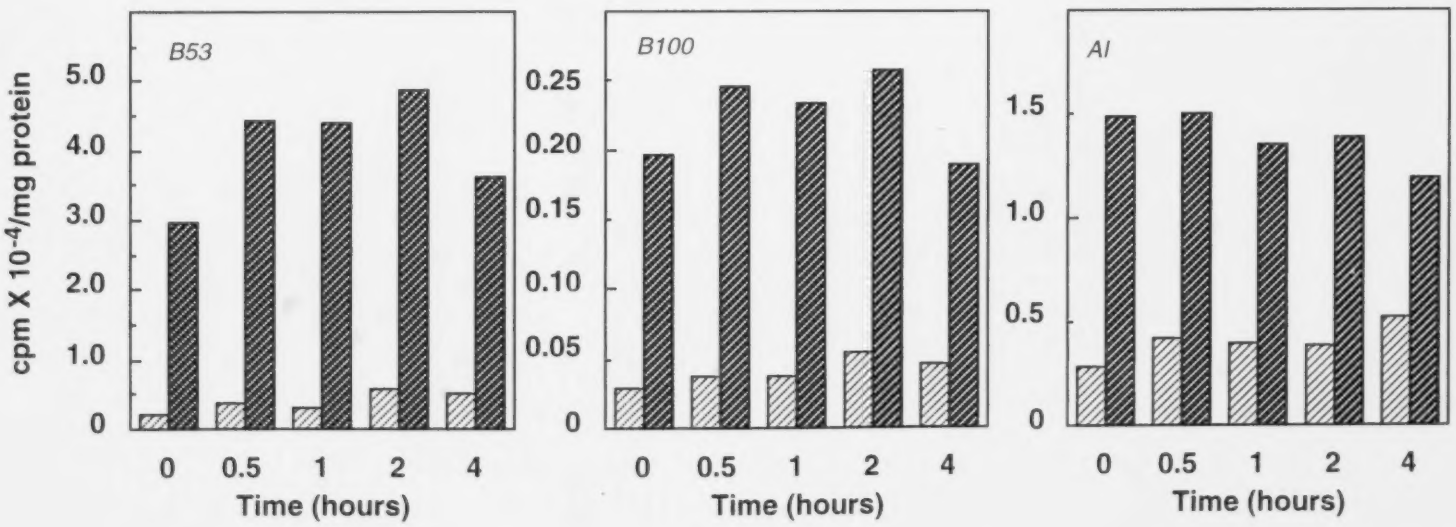


**Table IV.1:** Steady state levels of intracellular and medium lipids in stably transfected cell lines expressing different levels of apoB53. Cells (100 mm dishes) were incubated in serum free DMEM for 17 hours. Lipids were extracted from cells and media and quantified as described in chapter II. The values of intracellular lipids and medium phosphatidylcholine (PC) are the average of two determinations, and the values of medium triglyceride (TG) and total cholesterol (TC) are mean ± S. D. (n=3). The values in parentheses are the percentages of errors from the means. Repetition of the experiments produced similar results.

| Cell Line | Protein | Intracellular Lipids | | | Medium Lipids | | |
|---|---|---|---|---|---|---|---|
| | | TG | TC | PC | TG | TC | PC |
| | *mg/dish* | *µg/mg protein* | | | *µg/mg protein* | | |
| B53-1 | 2.72 | 22.7 (12%) | 19.9 (3.5%) | 36.3 (11%) | 2.55±0.72 | 2.56±0.18 | 7.72 (5.8%) |
| B53-14 | 3.58 | 27.0 (4.4%) | 19.0 (1.6%) | 35.3 (1.4%) | 1.17±0.17 | 2.64±0.11 | 6.37 (1.2%) |
| B53-10 | 3.30 | 30.5 (3.7%) | 18.4 (3.5%) | 34.4 (10%) | 0.94±0.16 | 1.90±0.19 | 3.23 (4.2%) |

# CHAPTER V. SUMMARY AND GENERAL DISCUSSION

Human apoB100 is synthesized in the liver and is the major protein component of VLDL, IDL, and LDL. ApoB100 is uniquely suited to its role in hepatic assembly of VLDL. Lipid binding regions alternate with hydrophilic segments throughout the apoB molecule. The extensive lipid binding regions account for its unique insolubility in aqueous media and inability to exchange between lipoprotein fractions in the plasma such as do the exchangeable apolipoproteins.

The role of the carboxyl terminus of apoB in the production of apoB-containing lipoproteins has been investigated using McA-RH7777 cell lines stably expressing human carboxyl truncated apoB64, apoB72 and full length apoB100. The data demonstrated that the relationship whereby the length of apoB determines the density of the lipoprotein produced holds for regions of apoB100 ranging from apoB23 to apoB53 and from apoB64 to apoB90. This result suggested that the density-length relationship may only apply to regions rich in amphipathic β strands. Therefore, it is proposed that during the fundamental stages of apoB-containing lipoprotein assembly, the β strand regions could confer the initial density of the lipoprotein. A second step may then occur in which the flexible amphipathic α helices could accommodate the fusion of a lipid droplet to result in the formation of VLDL for export from the cell. The data also showed that secretion of apoB-containing lipoproteins is not impaired as a consequence of carboxyl truncation. Thus, although this system consisted of human apoB constructs

transfected into rat hepatoma cells, it could lend support to the idea that changes pre-translationally (*e. g.*, loss of apoB mRNA stability) or other mechanisms may be responsible for low levels of apoB observed *in vivo*.

In chapter IV of this thesis, the contribution made by changes in levels of apoB mRNA on lipoprotein secretion was determined using stably transfected McA-RH7777 cell lines expressing different levels of human apoB53 mRNA. The results indicated that in these cells, the amount of human apoB secreted, the efficiency of human apoB secretion, and the amount of triglyceride secreted (and possibly synthesized), paralleled the level of human apoB synthesis; *i. e.*, as more apoB is synthesized, more apoB-containing lipoproteins are secreted. Since the density of both the human and endogenous rat lipoproteins were not altered, increased secretion of human apoB53 did not reduce the amount of lipid per lipoprotein particle nor did it occur at the expense of endogenous rat lipoprotein assembly and secretion. There was a reduction in steady state levels of secreted rat apoB100. The reason for this reduction is not entirely clear but could not be attributed to the level of human apoB53 expression. Therefore, the availability of lipids may not be the sole limiting factor that governs the efficiency of apoB secretion. Rather, the level of apoB expression may directly stimulate the synthesis and secretion of apoB-containing lipoproteins.

Stably transfected McA-RH7777 cell lines expressing carboxyl truncations of human apoB have been invaluable to the study of structure-function relationships of various aspects of apoB metabolism. Information

on the role that various of apoB play in lipoprotein assembly and secretion as well as on the contribution that changes in mRNA levels make are but a few of the uses of these cell lines. The interplay between apoB, lipids and other factors during the lipoprotein assembly process is only beginning to be discerned for which these cell lines may prove useful.

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